LUÍS MIGUEL MOITA DE CARVALHO

ASPECTOS ECOLÓGICOS DAS MICORRIZAS ARBUSCULARES NA VEGETAÇÃO DE SAPAL

Faculdade de Ciências da Universidade de Lisboa

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À Inês e
aos meus pais

Declaração

Na elaboração desta dissertação foi feito o aproveitamento total de resultados de trabalhos já publicados ou submetidos para publicação, que constituem alguns capítulos da presente tese. Uma vez que estes trabalhos foram realizados em colaboração, e de acordo com o disposto no nº 1 do Artigo 15º do Regulamento de Doutoramento da Universidade de Lisboa, publicado no Diário da República – II Série Nº 194, de 19-08-1993, o candidato esclarece que participou na obtenção, análise e discussão dos resultados, bem como na redacção dos manuscritos.

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Luís Miguel Moita de Carvalho

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ÍNDICE

RESUMO

Os sapais são ecossistemas caracterizados pela inundação regular com água do mar. O alagamento e a salinidade são os principais factores que influenciam a vegetação nos sapais. A maioria das plantas de sapal possui adaptações morfológicas e fisiológicas que lhes permitem sobreviver neste tipo de habitat. A formação de micorrizas arbusculares (AM) poderá constituir também uma importante estratégia de adaptação das plantas às condições adversas dos sapais. No entanto, o conhecimento das AM nos sapais ainda é escasso. Este trabalho teve como principal objectivo contribuir para um maior conhecimento da ecologia das AM nos sapais. Especificamente, procurou caracterizar a ocorrência e distribuição das AM, investigar a adaptação dos fungos AM às condições de alagamento e salinidade, e avaliar a contribuição das AM na tolerância das plantas ao alagamento salino por acção das marés.

Num sapal do estuário do Tejo, a avaliação da colonização em plantas demonstrou a ocorrência de AM nas zonas menos e mais alagadas do sapal e uma reduzida proporção de espécies vegetais formando AM. *Aster tripolium* foi a única espécie vegetal micotrófica presente nas duas zonas. A colonização apenas se relacionou com a fenologia das plantas na zona menos alagada. A diversidade de fungos AM encontrada foi igualmente muito baixa, com predominância da espécie *Glomus geosporum*. Nas duas zonas do sapal os sedimentos possuíam potencial de inóculo AM suficiente para iniciar a colonização das plantas. A distribuição dos esporos de fungos AM, um dos principais tipos de propágulos, relacionou-se principalmente com a distribuição das plantas hospedeiras AM, originando zonas de infectividade no solo do sapal. Os resultados destas experiências mostraram que a presença e distribuição das AM em sapais parecem ser mais determinadas pela identidade das espécies vegetais e pela distribuição das plantas hospedeiras, do que por factores abióticos. A germinação dos esporos de fungos AM nativos do sapal, única fase do ciclo de vida destes fungos em que a presença da planta não é obrigatória, não foi afectada por níveis elevados de salinidade e por níveis de água no solo acima da capacidade de campo, demostrando a tolerância destes fungos à salinidade e ao alagamento. A infectividade dos fungos AM nativos de sapal, isto é, a sua capacidade de infectar as raízes, foi mais afectada por níveis elevados de salinidade do que pelo alagamento permanente ou regular simulando a acção da maré. Por outro lado, a actividade metabólica dos fungos AM não foi influenciada por condições de alagamento, sugerindo que a funcionalidade dos fungos na simbiose não é afectada por essas condições. De facto, os fungos AM aumentaram a tolerância das plantas de *A. tripolium* da

população do estuário do Tejo ao alagamento salino por acção da maré ao atenuarem os efeitos negativos no crescimento das plantas. Nestas plantas não se verificaram benefícios em condições de ausência de alagamento. O crescimento das plantas de *A. tripolium* provenientes de outra população estudada (estuário de Westerschelde, Holanda) não foi afectado pelo alagamento com água do mar e as plantas não apresentaram benefícios evidentes das AM. A aquisição de azoto pelas plantas parece ter sido aumentada pela presença de fungos AM, independentemente do regime de alagamento e da população de *A. tripolium.* Os efeitos benéficos das AM no crescimento de *A. tripolium* parecem ser mediados por factores abióticos e dependentes da população ou da capacidade de tolerância das plantas a esses factores.

Os resultados deste trabalho mostraram que a adaptação dos fungos AM à salinidade e ao alagamento é um mecanismo explicativo da sua persistência nos sapais, e que as AM constituem um factor importante na tolerância de algumas plantas de sapal ao alagamento salino periódico por acção da maré.

Palavras-chave: alagamento, micorrizas arbusculares, salinidade, sapais, tolerância.

ABSTRACT

Salt marshes are ecosystems characterized by the regular inundation with seawater. Flooding and salinity are the main factors influencing salt marsh vegetation. The majority of the salt marsh plants have morphological and physiological adaptations allowing them to survive in this type of habitat. The formation of arbuscular mycorrhizas (AM) can be another important adaptation strategy of plants to the adverse conditions of salt marshes. However, the knowledge of AM in salt marshes is scarce. The main objective of this work was to contribute to a higher knowledge of the ecology of AM in salt marshes. Specifically, it seeked to characterize the occurrence and distribution of AM, to investigate AM fungal adaptation to flooding and salinity conditions, and to evaluate the contribution of AM in plant tolerance to seawater tidal flooding.

In a salt marsh of the Tagus estuary, the assessment of plant colonization showed AM occurrence in less and more flooded zones of the marsh and a low proportion of AM plant species. *Aster tripolium* was the only AM species occurring in both zones. AM colonization was only related to plant phenology in the less flooded zone. A low AM fungal diversity was also found with *Glomus geosporum* as the dominant species. The sediments of both marsh zones had sufficient AM inoculum potencial to initiate plant colonization. The distribution of AM fungal spores, one of the main propagules types, was mainly related to the distribution of AM plant hosts, creating infectivity zones in the salt marsh soil. The results of these experiments showed that the occurrence and distribution of AM in salt marshes are likely more determined by plants species identity and host plant distribution than by abiotic factors. High levels of salinity and soil water levels above field capacity did not affect spore germination of salt marsh indigenous AM fungi, which is the only host independent life cycle stage of these fungi, showing their tolerance to salinity and flooding. The infectivity of salt marsh indigenous AM fungi, that is, their ability to infect roots, was more affected by high salinity levels than by tidal or continuous flooding. The activity of AM fungi was not affected by flooding suggesting that the fungal functionality in the symbiosis is not affected by those conditions. In fact, AM fungi improved the tolerance of *A. tripolium* plants from the Tagus estuary population to seawater tidal flooding mitigating the negative effects in plant growth. Mycorrhizal benefits were not found in these plants under non-flooding conditions. The growth of *A. tripolium* plants from another studied population (Westerschelde estuary, The Netherlands) was neither affected by tidal flooding nor improved by AM. AM fungi likely

improved plant nitrogen acquisition, regardless of watering regime and *A. tripolium* population. The mycorrhizal benefits in *A. tripolium* growth are likely mediated by abiotic factors and dependent on plant population or on plant tolerance ability to those factors.
The results of this study showed that the adaptation of AM fungi to salinity and flooding is a mechanism explaining their persistence in salt marsh, and that AM are an important factor in the tolerance of some salt marsh plants to seawater tidal flooding.

Key words: arbuscular mycorrhizas, flooding, salinity, salt marshes, tolerance.

CAPÍTULO 1

Introdução geral

Introdução geral

1.1. Sapais

1.1.1. Caracterização dos sapais

Os sapais, zonas costeiras húmidas colonizadas por plantas vasculares sujeitas a inundações periódicas com água salgada em consequência da acção das marés, encontram-se entre os ecossistemas mais produtivos do planeta (Bertness, 1992; Brotas, 1995; Mitsch & Gosselink, 2000). Surgindo em áreas de baixa energia protegidas da ondulação do mar, como estuários, sistemas lagunares e baías, os sapais ocorrem nas zonas temperadas e subpolares a norte do paralelo 30ºN e a sul do paralelo 30ºS (Barnes & Hughes, 1982; Day Jr. *et al*., 1988).

A hidrologia é o factor mais importante que condiciona os processos físicos, químicos e biológicos dos sapais (Mitsch & Gosselink, 2000). O ciclo hidrológico nos sapais é dominado maioritariamente pela amplitude das marés, mas também é influenciado por correntes oceânicas, processos de evaporação, ventos, fluxos de água fresca e eventos catastróficos. A periodicidade de inundação pela água da maré provoca variações temporais e espaciais em vários factores abióticos, incluindo a estrutura do sedimento, o potencial redox, a salinidade e os níveis de nutrientes (Oenema *et al*., 1988).

A formação dos sapais está directamente relacionada com o ciclo hidrológico e a dinâmica da vegetação. O solo do sapal desenvolve-se através da deposição de sedimentos e matéria orgânica transportados pelas águas fluviais e marinhas, devido à acção das marés, e também pela acumulação de detritos orgânicos da vegetação (Adam, 1990; Kastler & Wiberg, 1996; Cahoon *et al*., 1999). A vegetação colonizadora contribui para a acumulação de sedimentos, influenciando a consolidação do solo e a topografia dos sapais ao elevar a superfície no sentido mais distante da zona intertidal (Caçador & Vale, 2002). O ciclo das marés controla a frequência e intensidade da inundação do solo e da vegetação: as marés de menor amplitude apenas inundam as cotas mais baixas do sapal, enquanto que marés de maior amplitude podem inundar cotas mais elevadas. O tempo de submersão diminui à medida que a elevação da superfície do sapal aumenta, delineando, geralmente, duas áreas: a zona baixa (limite inferior do sapal), alagada uma ou duas vezes ao dia e a zona alta (limite superior do sapal), alagada menos frequentemente (Rozema *et al*., 1985; Callaway *et al*., 1990; Bakker *et al*., 1993). As zonas altas podem também ser inundadas quando surgem precipitações elevadas, diluindo consideravelmente a salinidade (Caçador & Vale, 2002).

As águas fluviais e das marés são também fontes importantes de nutrientes para os sapais que os retêm, tais como, azoto, fósforo, cálcio e magnésio (Valiela *et al*., 1973; Valiela & Teal, 1979; De Jonge, 1990; van Wijnen & Bakker, 2000). Devido à acção de ressuspensão das marés, os sapais funcionam como sistemas abertos, exportando também nutrientes e material orgânico para as águas estuarinas e costeiras, contribuindo, assim, de forma extremamente importante para a cadeia alimentar que suporta muitos organismos marinhos (Adam, 1990; Cabrita, 1997). Os sapais desempenham também funções depuradoras ao incorporarem e armazenarem nos sedimentos, metais pesados dissolvidos ou associados a partículas em suspensão na água (Oenema *et al*., 1988; Orson *et al*., 1992; Caçador, 1994). Esta acção é de valor ecológico muito importante pois, localizando-se, geralmente, na periferia de áreas de influência antropogénica, os sapais estão sujeitos a descargas de poluentes (Huiskes & Rozema, 1988; Otte, 1991). A vegetação desempenha papel relevante nesta acção depuradora ao aprisionar os metais no sistema radicular e ao retê-los nos seus tecidos (Alberts *et al*., 1990; Otte, 1991; Caçador *et al*., 1996, 2000).

O alagamento e a baixa drenagem originam condições de hipoxia (baixa disponibilidade de oxigénio) nos solos, devido à baixa solubilidade (0.28 mol m^{-3} a 20ºC) e taxa de difusão do oxigénio na água (10 000 vezes menor que no ar) e ao rápido uso do oxigénio dissolvido pelas bactérias e raízes (Grable, 1966). Os solos saturados apresentam condições típicas de anaerobiose com potenciais redox baixos (Howes *et al*., 1981; Armstrong *et al*., 1985) e acumulação de sulfuretos e de formas reduzidas dos elementos ferro e manganês (Armstrong, 1975; Devai & DeLaune, 1995). A frequência e a intensidade das marés associadas à topografia e elevação da superfície dos sedimentos conduzem à variação espacial e temporal do conteúdo em água e do potencial redox dos solos, com solos mais saturados e mais redutores na zona baixa do sapal (Armstrong *et al*., 1985; Bertness & Ellison, 1987; Pennings & Callaway, 1992; Ewing *et al*., 1997).

A inundação pelas marés com água do mar conduz a solos com elevados níveis de cloreto de sódio e de outros sais presentes na água do mar, originando elevados níveis de salinidade (Callaway *et al*., 1990; Pennings & Callaway, 1992; Hacker & Bertness, 1999). A combinação do regime das marés, da elevação, da evaporação e da precipitação determina a variação espacial e temporal dos níveis de salinidade no sapal. De modo geral, na zona baixa do sapal a regularidade de inundação estabiliza os níveis de salinidade que são normalmente elevados e muitas vezes próximos dos da salinidade da água do mar, enquanto que na zona alta existe maior variação no teor em sal devido à menor frequência de inundação, tendo maior influência as variáveis climáticas e o fluxo de água no solo (Callaway *et al*., 1990;

Pennings & Callaway, 1992; Mitsch & Gosselink, 2000). Nos sapais de clima Mediterrânico a evapotranspiração no verão aumenta a concentração dos sais no solo, enquanto a pluviosidade no inverno a faz diminuir (Callaway *et al*., 1990).

1.1.2. Vegetação dos sapais

Os sapais são habitats particularmente adversos para as plantas vasculares, limitando a presença às espécies capazes de tolerar as condições edáficas extremas: elevada salinidade, inundação frequente, condições de hipoxia, potenciais redox baixos, acumulação de fitotoxinas (enxofre, ferro e manganês nos seus estados reduzidos) e baixa disponibilidade de azoto (Armstrong *et al*., 1985; Howes *et al*., 1986; Mendelssohn & McKee, 1988; Armstrong *et al*., 1991; Rozema & van Diggelen, 1991; Bertness *et al*., 1992a,b; Pennings & Callaway, 1992; Chambers *et al*., 1998; Levine *et al*., 1998; Sánchez *et al*., 1998; Hacker & Bertness, 1999; van Wijnen & Bakker, 1999; Huckle *et al*., 2000). Os sapais possuem, consequentemente, baixa diversidade vegetal, sendo constituídos por espécies halófitas pertencentes, na sua maioria, às famílias Asteraceae, Caryophyllaceae, Chenopodiaceae, Cyperaceae, Juncaceae, Juncaginaceae, Plantaginaceae, Plumbaginaceae, Poaceae e Primulaceae.

A frequência e intensidade da maré determina, geralmente, a distribuição das plantas no sapal formando zonas específicas distintas, geralmente, ao longo de um gradiente de elevação (Vince & Snow, 1984; Pennings & Moore, 2001). Este padrão de zonação tem sido referenciado como estando relacionado com variações nas condições edáficas associadas com o alagamento pelas marés, nomeadamente com os gradientes das concentrações de oxigénio e sal (Howes *et al*., 1981; Cooper, 1982; Armstrong *et al*., 1985; Callaway *et al*., 1990). No entanto, para além dos factores abióticos, vários estudos têm também salientado a importância dos processos interespecíficos na determinação da zonação das plantas nos sapais (Bertness & Ellinson, 1987; Bertness, 1991a,b; Pennings & Callaway, 1992; Levine *et al*., 1998; Hacker & Bertness, 1999; Pennings & Moore, 2001). Assim, nas zonas baixas dos sapais, onde existem maiores condições de hipoxia e níveis geralmente elevados de salinidade, a zonação parece resultar da tolerância das espécies aos factores edáficos, enquanto que nas zonas altas, onde ocorrem condições mais arejadas e níveis de salinidade geralmente mais baixos, a competição interespecífica parece ser determinante para o padrão de distribuição das plantas.

As plantas dos sapais apresentam adaptações morfológicas, bioquímicas e fisiológicas em resposta às limitações impostas pelas condições de alagamento e salinidade dos solos. Em

condições de redução de oxigénio disponível no solo, a sobrevivência das plantas é, em parte, dependente de um eficiente transporte de oxigénio da parte aérea para as raízes permitindo a respiração aeróbia e a oxidação das fitotoxinas quimicamente reduzidas (Burdick & Mendelssohn, 1990; Naidoo *et al*., 1992). A presença de aerênquima, tecido especializado de parênquima com largos espaços intercelulares que permitem uma rápida difusão de oxigénio para as raízes, constitui uma das principais adaptações morfológicas de várias espécies presentes nos sapais (Justin & Armstrong, 1987; Burdick & Mendelssohn, 1990; Armstrong *et al*., 1991; Naidoo *et al*., 1992). O desenvolvimento de raízes superficiais, o aumento da razão radicular área : volume radicular, uma menor respiração das raízes e a estimulação da actividade da enzima ADH (álcool desidrogenase) podem também contribuir para diminuir os efeitos do alagamento (Armstrong *et al*., 1991; Naidoo *et al*., 1992). O alongamento das folhas é um mecanismo de resposta das plantas à redução da taxa fotossintética devido à periódica submersão da parte aérea e à indução do fecho estomático pelo alagamento (Gleason & Zieman, 1981; Ernst, 1990).

A elevada salinidade presente nos solos dos sapais tem consequências negativas para as plantas afectando a osmorregulação, a fotossíntese e a respiração, provocando toxicidade (ao nível de processos membranares, enzimáticos e de tomada de iões, como nitrato, potássio e cálcio) e alterando o balanço iónico (Pezeshki *et al*., 1987; Shannon *et al*., 1994). A existência de glândulas epidérmicas especializadas na exclusão de sais (Bradley & Morris, 1991), a limitação da absorção radicular de sais (Smart & Barko, 1980), a suculência diluindo a concentração de sais e permitindo a entrada de água na raiz (Cavalieri, 1983) e a alteração na composição membranar (Wu *et al*., 1998) são mecanismos de adaptação apresentados pelos halófitos presentes nos sapais.

Do ponto de vista nutricional, a vegetação dos sapais está sujeita à baixa disponibilidade de N que, devido à saturação dos solos, está, na sua maioria, sob a forma de azoto orgânico (Valiela & Teal, 1974; Patrick & DeLaune, 1976; Mendelssohn, 1979; Kiehl *et al*., 1997; Levine *et al*., 1998; Cartaxana, 1999; van Wijnen & Bakker, 1999). Pelo contrário, o fósforo não é usualmente um factor limitante nos sapais uma vez que está presente em quantidades relativamente elevadas na forma disponível, devido às condições reduzidas dos sedimentos (Valiela & Teal, 1974; Patrick & DeLaune, 1976; Ponnamperuma, 1979).

1.1.3. Os sapais do estuário do Tejo

O estuário do rio Tejo é dos maiores da Europa Ocidental localizando-se junto à área metropolitana de Lisboa. Ocupa uma área total aproximada de 320 km^2 com uma área intertidal de 136 km^2 e 20 km^2 de área ocupada por sapais (Fig. 1.1). O estuário apresenta uma assimetria entre as suas margens, sendo a direita rectilínea e a esquerda muito recortada. O regime de marés é do tipo semi-diurno incluindo-se na classe dos estuários mesotidais, isto é, a amplitude de maré varia entre 2 m e 4,6 m. A salinidade depende dos volumes de massa de água doce e de água salgada, variando entre 5‰ a montante e 36‰ próximo de Lisboa. O clima do estuário do Tejo é do tipo Mediterrânico, caracterizado por duas estações temperadas, o Outono e a Primavera, intercaladas com duas estações extremas: uma quente e seca, o Verão, e outra fria e húmida, o Inverno. A precipitação média anual situa-se entre 600 mm e 700 mm (Caçador, 1994; Costa, 1999).

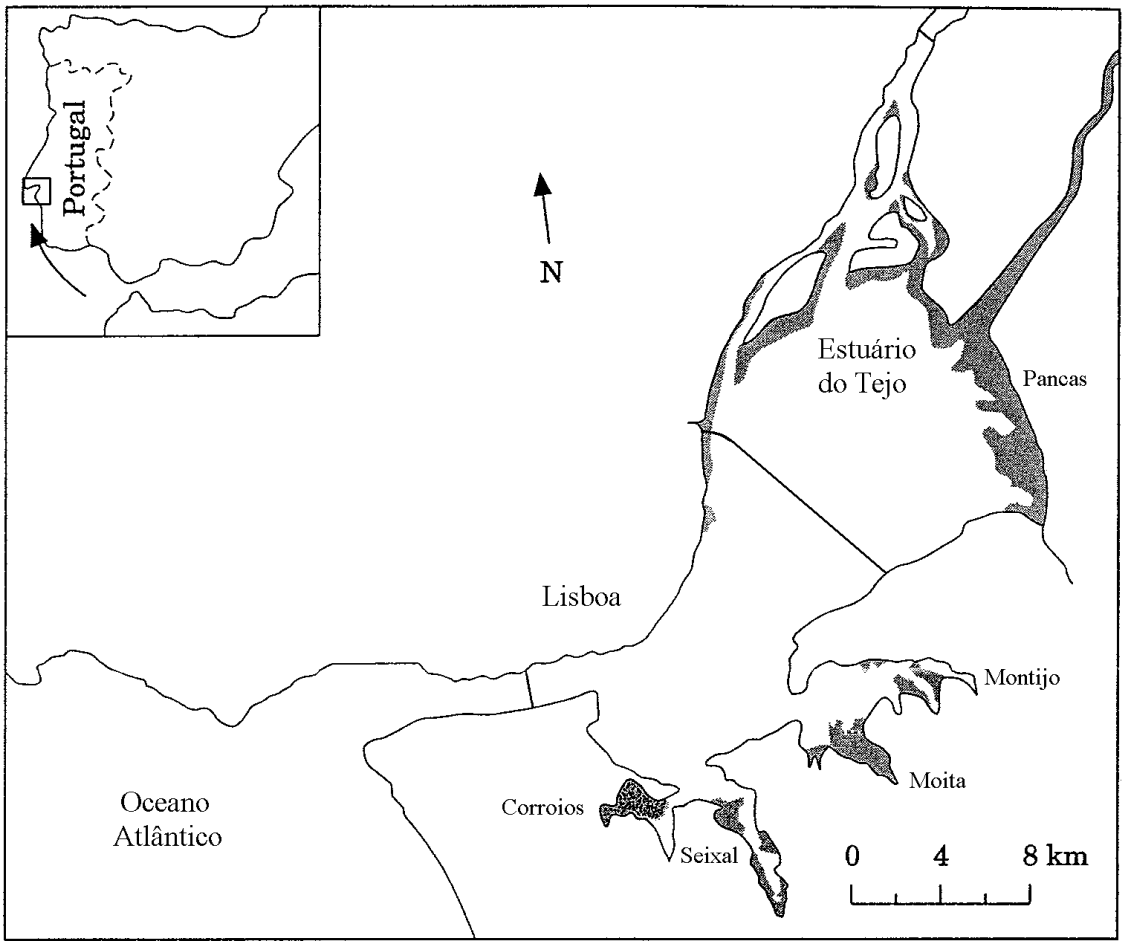

Fig. 1.1. Estuário do Tejo. A sombreado estão assinaladas as manchas de sapal.

Os sapais localizam-se essencialmente na margem esquerda do estuário do Tejo, embora também se situem nos mouchões, nas ilhotas e, muito limitadamente, na margem direita (Fig. 1.1). A principal mancha de vegetação contínua localiza-se entre o mouchão das Garças e Alcochete, englobando o sapal de Pancas (38º49´N 08º57´W). Nesta vasta extensão de sapal encontram-se múltiplos canais e esteiros devido ao repetido avanço e escoamento das águas das marés. Os sapais do estuário do Tejo confinados com terrenos agrícolas nas cotas mais elevadas estão sujeitos a descargas de efluentes agrícolas, e os mais próximos das áreas industriais e metropolitanas de efluentes industriais contaminados com metais pesados.

A vegetação dos sapais do estuário do Tejo é constituída por espécies herbáceas e espécies arbustivas de pequeno porte, sendo as espécies mais significativas: *Spartina maritima* (Curtis) Fernald, *Scirpus maritimus* L., *Halimione portulacoides* (L.) Aellen (sinónimo *Atriplex portulacoides* L.), *Arthrocnemum fruticosum* (L.) Moq. (sinónimos *Sarcocornia fruticosa* (L.) Scott e *Salicornia fruticosa* (L.) L.), *Arthrocnemum perenne* (Miller) Moss (sinónimos *Sarcocornia perennis* (Miller) Scott e *Salicornia perennis* Miller), *Salicornia nitens* P.W. Ball & Tutin, *Aster tripolium* L., *Puccinellia maritima* (Hudson) Parl., *Phragmites communis* (Cav.) Trin ex Steudel e *Inula chritmoides* L. (Ramos & Catarino, 1979; Caçador 1986, 1994). Destas espécies *Spartina maritima*, espécie pioneira na colonização das vasas consolidadas, *Halimione portulacoides*, *Arthrocnemum fruticosum* e *Arthrocnemum perenne* predominam, respectivamente, no sentido das cotas crescentes dos sapais (Catarino & Caçador, 1991).

1.2. Micorrizas

As associações simbióticas entre plantas e microorganismos do solo desempenham funções extremamente importantes tanto ao nível do indivíduo como do ecossistema (Martins-Loução, 2002). As micorrizas são associações estabelecidas entre raízes das plantas e fungos do solo, ocorrendo na maioria dos ecossistemas e em aproximadamente 80 a 90% das espécies vegetais (Harley & Smith, 1983; Trappe, 1987; Smith & Read, 1997). As espécies que não formam micorrizas em qualquer estado do seu ciclo de vida e em quaisquer condições ambientais pertencem na sua maioria às famílias Amaranthaceae, Brassicaceae, Caryophyllaceae, Chenopodiaceae e Cyperaceae (Tester *et al*., 1987). De modo geral, as micorrizas caracterizam-se por serem mutualismos em que a planta hospedeira recebe do

fungo nutrientes minerais e o fungo obtém hidratos de carbono provenientes da planta (Harley & Smith, 1983; Allen, 1991; Smith & Read, 1997).

Segundo Read (2002), as micorrizas podem classificar-se em vários tipos de acordo com as características dos fungos envolvidos e as características morfológicas das associações (Gonçalves, 2000): arbusculares, ectomicorrizas, arbutóides, monotropóides, ericóides e orquidáceas. De entre os vários tipos as micorrizas arbusculares (AM*) são as mais abundantes: cerca de 60% das espécies vegetais formam este tipo de micorriza em *taxa* e habitats diversificados (Newman & Reddell, 1987; Trappe, 1987).

As AM são formadas por fungos pertencentes à ordem Glomales da classe Glomeromycetes, divisão Glomeromycota (Schüssler *et al*., 2001). Apesar de estabelecerem associações com grande diversidade de espécies vegetais, o número de espécies fúngicas até agora descritos é restrito, cerca de 150, pertencentes a 6 géneros (Morton & Benny, 1990; Morton & Redecker, 2001). Os fungos AM caracterizam-se por terem hifas intraradiculares asseptadas, pelo menos enquanto novas, que ao penetrarem nas células da raiz formam estruturas funcionais de troca de elementos e compostos, os arbúsculos, e estruturas intra ou intercelulares de reserva, as vesículas. No solo os fungos AM formam geralmente uma extensa rede de hifas, micélio extraradicular, e esporos que, nalgumas espécies de fungos, também podem ser formados dentro da raiz. Os fungos AM possuem como forma de propagação os esporos, o micélio extraradicular e fragmentos de raízes infectadas (Smith & Read, 1997).

As AM podem apresentar diversos benefícios para as plantas. O melhoramento da nutrição mineral é o efeito mais estudado e mais evidente da simbiose. Através da sua rede de micélio o volume acessível de absorção aumenta contribuindo para a tomada selectiva de elementos pouco móveis como o fósforo (Koide, 1991; Jakobsen *et al*., 1992; Sanders & Fitter, 1992; Pearson & Jakobsen, 1993; Jakobsen, 1995; Moyersoen *et al*., 1998; Schweiger & Jakobsen, 1999), o cobre e o zinco (Kothari *et al*., 1991; Li *et al*., 1991; Azaizeh *et al*., 1995), e também de azoto inorgânico (Johansen *et al*., 1992, 1993; Tobar *et al*., 1994a,b; Hawkins & George, 1999; Azcón *et al*., 2001; Cruz *et al*., 2003). Recentemente, surgiram evidências de que as AM podem também contribuir para alguma tomada de azoto orgânico pelas plantas (Hodge *et al*., 2001). Segundo alguns autores as AM podem favorecer a aquisição de água, embora este ponto seja controverso (Allen, 1982; Hardie, 1985; Ruiz-Lozano & Azcón, 1995). Outras funções reconhecidas às micorrizas, por vezes consideradas como estando associadas ao

* AM – designação inglesa, internacionalmente adoptada, que significa *arbuscular mycorrhiza(s).*

melhoramento da nutrição mineral, são o seu potencial para fornecer protecção às plantas contra organismos patogénicos (Benhamou *et al*., 1994; Newsham *et al*., 1994, 1995; Norman *et al*., 1996; Cordier *et al*., 1998; Pozo *et al*., 2002), para aumentar a tolerância das plantas à salinidade (Ruiz-Lozano *et al*., 1996; Hatimi, 1999; Tsang & Maun, 1999; Al-Karaki, 2000; Ruiz-Lozano & Azcón, 2000; Cantrell & Linderman, 2001; Yano-Melo *et al*., 2003), à seca (Allen & Allen, 1986; Augé *et al*., 1994; Ruiz-Lozano *et al*., 1995; Subramanian *et al*., 1995; Ebel *et al*., 1996) e a metais pesados (Dehn & Schüepp, 1989; Heggo *et al*., 1990; Joner & Leyval, 1997; Hildebrandt *et al*., 1999; Davies *et al*., 2001). As AM podem também contribuir para a estabilização dos agregados do solo (Andrade *et al*., 1998; Jastrow *et al*., 1998; Rilling *et al*., 1999; Bearden & Petersen, 2000).

Os efeitos directos das AM nas plantas traduzem-se, geralmente, no aumento da biomassa aérea e na alteração da forma radicular e foliar, e/ou em maior fecundidade e vitalidade (Smith & Read, 1997; Martins-Loução, 2002). No entanto, o grau de resposta, isto é, o grau de dependência micorrízica das plantas varia entre as espécies vegetais (Janos, 1980; Brundrett 1991). Existem espécies em que a associação é obrigatória, sendo dependentes da micorriza para a sua sobrevivência, outras em que a associação é facultativa, melhorando o seu desenvolvimento na presença da micorriza, noutras a resposta à micorriza é positiva apenas sob certas condições ambientais e existem, ainda, espécies que não formam associações do tipo micorriza. Ao nível do indivíduo, a resposta da planta à micorriza deriva do balanço entre custos (hidratos de carbono encaminhados para o fungo) e benefícios (geralmente, aquisição de nutrientes). Marschner & Dell (1994) estimaram que as hifas dos fungos AM podem contribuir até cerca de 80% e 25% das necessidades de uma planta em fósforo e azoto, respectivamente. Por outro lado, entre 5 a 20% de todo o carbono fotossintetizado pela planta pode ser consumido pelo fungo AM (Jakobsen & Rosendahl, 1990).

A relação entre custos e benefícios, e consequentemente a resposta da planta à micorriza, pode depender das características dos simbiontes (planta hospedeira e fungo), e das condições edáficas e ambientais do habitat (Brundrett, 1991; Francis & Read, 1995; Johnson *et al*., 1997; Jakobsen *et al*., 2002). Estudos recentes têm demonstrado que as espécies de plantas podem diferir nas suas respostas a espécies ou isolados individuais de fungos AMF (Streitwolf-Engel *et al*., 1997; van der Heijden *et al*., 1998a,b), que as respostas dos fungos podem variar consoante a espécie hospedeira (Johnson *et al*., 1992; Bever *et al*., 1996; Eom *et al*., 2000) e que morfotipos semelhantes de fungos AM de diferentes habitats podem conferir benefícios diferentes à mesma espécie hospedeira (Allen *et al*., 1995). Esta

multifuncionalidade das AM permite explicar o papel relevante que possuem ao nível do ecossistema, onde podem determinar a diversidade, estrutura e a produtividade das comunidades vegetais (Grime *et al*., 1987; Gange *et al*., 1993; Sanders & Koide, 1994; van der Heijden *et al*., 1998a,b; Hartnett & Wilson, 1999; Klironomos *et al*., 2000; O'Connor *et al*., 2002).

As condições edáficas e ambientais podem influenciar o comportamento do fungo e a resposta da simbiose. Apesar de os fungos AM tenderem a ser generalistas, a sua funcionalidade pode variar consoante o tipo de habitat. Exemplos de factores do solo ou ambiente com capacidade de influenciar a efectividade da simbiose são: disponibilidade de nutrientes, arejamento, salinidade, disponibilidade hídrica e toxicidade do solo, intensidade luminosa, infecções por organismos patogénicos e interacções com bactérias rizosféricas (Brundrett 1991, Jakobsen *et al*., 2002). Existem evidências que sugerem a adaptação de espécies ou isolados de fungos AM locais a determinadas condições edáficas próprias do seu habitat (ver Brundrett, 1991). Por exemplo, alguns trabalhos indicam que ecótipos de fungos AM de locais contaminados apresentam maior tolerância a metais pesados do que isolados de fungos AM de locais não contaminados (Gildon & Tinker, 1983; Weissenhorn *et al*., 1993, 1994; del Val *et al*., 1999). As adaptações ambientais dos fungos AM podem ser determinadas pelo hospedeiro mas podem também resultar da fisiologia e genética dos próprios fungos (Allen *et al*., 1995). A tolerância dos fungos AM a condições existentes no seu habitat pode fornecer uma base para explicar a persistência destes organismos em habitats específicos.

1.2.1. Micorrizas arbusculares nos sapais

Os sapais são ambientes adversos para os fungos AM. Os fungos AM são organismos obrigatoriamente aeróbios e foram considerados fracamente adaptados a ambientes alagados (Mosse *et al*., 1981; Sylvia & Williams, 1992). Condições de alagamento dos solos com baixos níveis de oxigénio e de potencial redox podem limitar o crescimento dos fungos e o desenvolvimento da colonização em raízes de plantas hospedeiras (Saif, 1981, 1983; Tanner & Clayton, 1985; Rozema *et al*., 1986; Khan & Belik, 1995; Miller & Bever, 1999; Miller & Sharitz, 2000), o número de esporos de fungos AM no solo (Anderson *et al*., 1984; Khan, 1993) e a sua capacidade de germinação (LeTacon *et al*., 1983; Sylvia & Schenck, 1983). Saif (1981, 1983) observou que o efeito da baixa disponibilidade de oxigénio no desenvolvimento da colonização pode ser dependente da espécie de fungo AM e da identidade da planta

hospedeira. A infecção por fungos AM pode também ser dificultada nestes solos pelo decréscimo da permeabilidade das membranas citoplasmáticas com a saturação do solo (Janick *et al*., 1981). As micorrizas podem também, eventualmente, ser inibidas pela acumulação dos produtos reduzidos tóxicos resultantes do metabolismo anaeróbio em sedimentos muito reduzidos (Mosse *et al*., 1981). A salinidade em concentrações altas pode ter impacto negativo nas micorrizas, inibindo o crescimento dos fungos e afectando a colonização (Kim & Weber, 1985; Pfeiffer & Bloss, 1988; Juniper & Abbott, 1993; McMillen *et al*., 1998; Al-Karaki, 2000; Cantrell & Linderman, 2001; Johnson-Green *et al*., 2001; Pande & Tarafdar, 2002). Uma forte redução na germinação de esporos de fungos AM com concentrações altas de sais de sódio e cloro tem sido observada em alguns trabalhos (Hirrel, 1981; Estaun 1989). Por outro lado, a germinação dos fungos e a sua capacidade de colonizar as raízes das plantas hospedeiras é amplamente afectada por níveis elevados de fósforo no solo (Graham *et al*., 1981; Miranda & Harris, 1994), que ocorrem geralmente nos sapais (Valiela & Teal, 1974; Patrick & DeLaune, 1976).

Apesar de todo este ambiente adverso, vários estudos têm demonstrado a existência de AM em plantas de sapal (Tabela 1.1) e noutros tipos de ecossistemas alagados (Søndergåard & Laegåard, 1977; Clayton & Bagyaraj, 1984; Tanner & Clayton, 1985; Wetzel & van der Valk, 1996; Cooke & Lefor, 1998; Miller, 2000) e consideravelmente salinos (Kim & Weber, 1985; Johnson-Green *et al*., 1995; Aliasgharzadeh *et al*., 2001; Landwehr *et al*., 2002). Muitas das espécies de sapal não formam micorrizas, embora tenha sido encontrada colonização por fungos AM nalgumas espécies de sapal pertencentes a famílias geralmente consideradas como não fazendo associações do tipo micorriza (Tabela 1.1). No entanto, em algumas dessas espécies não tem havido consistência no estado de colonização por AM, havendo autores que verificaram a presença de AM e outros que não encontraram qualquer colonização (Tabela 1.1). Estes resultados parecem indicar que talvez indique que a colonização nessas espécies pode depender das condições ambientais, fenológicas ou sazonais. Adicionalmente, a maioria dos trabalhos não observou, ou não analisou, a existência de arbúsculos nas raízes dessas espécies, estrutura exclusiva dos fungos AM e que, consequentemente, pode atestar da veracidade da presença de AM nas raízes e da funcionalidade da simbiose (Hirrel *et al*., 1978; Brundrett, 1991).

Tabela 1.1. Avaliação descrita na literatura da presença (+) ou ausência (-) de colonização de AM observada em espécies de plantas de sapal. -/+ indica espécie em que foi detectada ausência e presença de AM por diferentes autores

Família	Espécie	AM	Referências
Aizoaceae	*Sesuvium portulacastrum*	+	Sengupta & Chaudhuri, 1990
Apiaceae	*Apium nodiflorum*	-	Read *et al*., 1976
	Oenanthe lachenalii	+	Hildebrandt *et al*., 2001
Asteraceae	*Artemisia maritima*	+	van Duin *et al*., 1989; Hildebrandt *et al*., 2001
	Artemisia rupestris	+	Hildebrandt *et al*., 2001
	Aster tripolium	+	Mason, 1928; Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
	Iva frutescens	+	Cooke & Lefor, 1990
	Jaumea carnosa	+	Brown & Bledsoe, 1996; Hildebrandt *et al*., 2001
Brassicaceae	*Rorippa microphylla*	-	Read *et al*., 1976
Caryophyllaceae	*Sagina maritima*	+	Hildebrandt *et al*., 2001
	Spergularia maritima	-	Mason, 1928
	Spergularia marginata	-	Rozema *et al*., 1986; van Duin *et al*., 1989
	Spergularia salina	-/+	Hildebrandt *et al*., 2001
Chenopodiaceae	*Atriplex hastata*	-	Rozema *et al*., 1986
	Atriplex prostrata	+	van Duin *et al*., 1989
	Arthrocnemum indicum	+	Sengupta & Chaudhuri, 1990
	Cochlearia anglica	-	Rozema *et al*., 1986
	Cochlearia officinalis	+	Mason, 1928
	Halimione portulacoides	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989
	Salicornia brachystachya	-/+	Rozema *et al*., 1986
	Salicornia dolichostachya	+	Rozema *et al*., 1986
	Salicornia europaea	-/+	Mason, 1928; Hildebrandt *et al*., 2001
	Suaeda maritima	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989; Sengupta & Chaudhuri, 1990
Cyperaceae	*Cyperus difformis*	-	Khan, 1974
	Cyperus eleusinoides	-	Khan, 1974
Juncaceae	*Juncus gerardii*	-/+	Mason, 1928; Rozema *et al*., 1986; Cooke & Lefor, 1990; Hildebrandt *et al*., 2001
	Juncus bufonius	-	Khan, 1974
	Juncus maritimus	-	Mason, 1928; Rozema *et al*., 1986
Juncaginaceae	*Triglochin maritimum*	-/+	Mason, 1928; Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
Plantaginaceae	*Plantago coronopus*	+	Mason, 1928; Hildebrandt *et al*., 2001
	Plantago maritima	+	Mason, 1928; Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
Plumbaginaceae	*Armeria maritima*	-/+	Mason, 1928; Rozema *et al*., 1986; Hildebrandt *et al*., 2001
	Limonium carolinianum	+	Cooke & Lefor, 1990
	Limonium vulgare	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
Poaceae	*Agropyron junceum*	+	Hildebrandt *et al*., 2001
	Agrostis alba	+	Mason, 1928
	Distichlis spicata	-/+	Cooke & Lefor, 1990; Cooke *et al*., 1993; Hoefnagels *et al*., 1993
	Elymus pycnanthus	+	van Duin *et al*., 1989
	Festuca rubra ssp. litoralis	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
	Glyceria maritima	+	Mason, 1928
	Glyceria plicata	+	Read *et al*., 1976
	Phalaris arundinacea	+	Read *et al*., 1976
	Phragmites australis	-	Cooke & Lefor, 1990; Burke *et al*., 2002
	Puccinellia distans	-/+	Hildebrandt *et al*., 2001
	Puccinellia maritima	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
	Poa trivialis	+	Read *et al*., 1976
	Porteresia coarctata	+	Sengupta & Chaudhuri, 1990
	Spartina alterniflora	-	Cooke & Lefor, 1990; Hoefnagels *et al*., 1993
	Spartina anglica	-/+	Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
	Spartina cynosuroides	+	Hoefnagels *et al*., 1993
	Spartina patens	+	Cooke & Lefor, 1990; Cooke *et al*., 1993; Hoefnagels *et al*., 1993; Burke *et al*., 2002
Primulaceae	*Glaux maritima*	+	Mason, 1928; Rozema *et al*., 1986; van Duin *et al*., 1989; Hildebrandt *et al*., 2001
Typhaceae	*Typha angustata*	-	Khan, 1974

A sobrevivência e persistência de fungos AM nos sapais poderá ser explicada por alguns mecanismos adaptativos capazes de evitar ou tolerar as condições de stress. Os fungos podem evitar a falta de oxigénio nos sedimentos concentrando-se nas porções mais oxigenadas do sistema radicular. Brown & Bledsoe (1996) constataram a existência de colonização AM nos feixes radiais do aerênquima radicular na espécie de sapal *Jaumea carnosa*, estando a maioria dos arbúsculos concentrados nas células do aerênquima. Por outro lado, as espécies com aerênquima, ao libertarem oxigénio das raízes, oxigenam a sua rizosfera (Armstrong *et al*., 1991), podendo deste modo permitir a presença de fungos AM nessa zona. Contudo, demonstrou-se que esta perda de oxigénio para a rizosfera apenas fornece uma pequena fracção do oxigénio rizosférico (Bedford *et al*., 1991; Howes & Teal, 1994). Embora a presença de aerênquima possa explicar a ocorrência de AM nalgumas espécies de sapal, não pode ser a única justificação, pois existem espécies sem aerênquima que possuem micorrizas e espécies com aerênquima que não formam qualquer tipo de associação micorrízica (Rozema *et al*., 1986; Khan & Belik, 1995).

Os fungos AM e a colonização podem também restringir-se às camadas superficiais mais oxigenadas dos sedimentos, uma vez que o potencial redox diminui com a profundidade (Good & Patrick, 1987). Apesar de Brown & Bledsoe (1996) terem observado um decréscimo na colonização das raízes de *Jaumea carnosa* e no número de esporos, com o aumento da profundidade, Cooke e colaboradores (1993) verificaram colonização nas raízes de *Spartina patens* e *Distichlis spicata* até profundidades elevadas (42 cm). Como explicação sugeriram que, através do aerênquima presente nestas espécies, foi transportado oxigénio suficiente para permitir o crescimento do fungo.

Os fungos AM podem contornar as dificuldades do ambiente dos sapais funcionando sazonalmente, estando activos durante períodos menos alagados (Liberta *et al*., 1983). Os resultados até à data são contraditórios quanto a esta hipótese. Num sapal da Holanda, van Duin e colaboradores (1989) observaram que a formação de arbúsculos ocorria apenas durante a Primavera, enquanto que num sapal da Califórnia, Brown & Bledsoe (1996) observaram maior incidência de arbúsculos durante os meses de inverno (período de menor crescimento activo das plantas) nas zonas menos alagadas e não encontraram variação sazonal na ocorrência de arbúsculos nas zonas mais alagadas.

Outra potencial explicação para a sobrevivência dos fungos AM nos sapais é a sua adaptação às condições ambientais. Se os fungos AM tolerarem bem as condições de alagamento e salinidade, não será de esperar grandes diferenças entre a sua presença e o grau de colonização entre a zona baixa (mais regularmente alagada) e a zona alta do sapal (menos

frequentemente alagada). Enquanto que Rozema e coloboradores (1986) confirmaram a presença de AM em halófitas tanto na zona baixa como na zona alta, van Duin *et al*. (1989), Cooke & Lefor (1990) e Hoefnagels e colaboradores (1993) verificaram que a presença de AM era praticamente limitada às espécies vegetais da zona alta do sapal. Uma análise quantitativa, sazonal e espacial, comparando a colonização da mesma espécie vegetal em ambas as zonas com semelhantes comunidades de fungos AM, poderá esclarecer se a colonização AM nos sapais é dependente dos factores edáficos. Até à data, que se conheça, apenas um trabalho realizado em sapais fez este tipo abordagem. Brown & Bledsoe (1996) verificaram, surpreendentemente, que o grau de colonização *de Jaumea carnosa* era significativamente maior nas zonas mais alagadas. Os autores atribuíram este resultado ao facto de na zona alta haver menor efeito "tampão" das condições edáficas pela acção das marés, comparativamente às zonas mais alagadas. No entanto, as diferenças entre os vários locais a nível da composição das comunidades de fungos AM podem também ter influenciado os níveis de colonização.

A adaptação das AM aos sapais, a existir, pode ser específica: algumas espécies de fungos AM podem apresentar um determinado grau de tolerância ao alagamento e à salinidade. Poucos trabalhos se têm dedicado à identificação da diversidade de espécies de fungos AM nos sapais. Os sapais parecem apresentar baixa diversidade e serem constituídos por espécies pertencendo maioritariamente a um único género, *Glomus* (Sengupta & Chaudhuri, 1990; Brown & Bledsoe, 1996; Hildebrandt *et al*., 2001). Hildebrandt e colaboradores (2001) verificaram que uma única espécie, *Glomus geosporum*, era largamente predominante em sapais da Europa Central, à semelhança do que se verifica noutro tipo de solos salinos da Hungria (Landwehr *et al*., 2002). Estas evidências sugerem que pode haver uma adaptação específica de fungos AM às condições adversas dos sapais. As características adaptativas dos fungos AM podem também estar relacionadas com a utilização de formas de propagação mais resistentes ao stress, como os esporos (Abbott & Robson, 1991). A informação relativa à tolerância de fungos AM dos sapais às condições de alagamento e salinidade é praticamente inexistente, exceptuando a avaliação da colonização de plantas recolhidas nos sapais.

A formação de micorrizas poderá constituir uma importante estratégia de adaptação de várias plantas a estes habitats. Mas, os benefícios das AM para as plantas de sapal ainda são muito pouco conhecidos. O aumento da nutrição mineral é o efeito benéfico das AM mais reconhecido na maioria dos sistemas. Nos sapais é pouco provável que as AM aumentem a aquisição de fósforo, visto este elemento não ser, geralmente, um factor limitante para as plantas nestes ecossistemas, tal como acontece noutros sistemas alagados. Por outro lado,

obteve-se evidência de que o crescimento e a tomada de fósforo pelas hifas dos fungos AM podem ser reduzidos em resposta a baixas concentrações de oxigénio no solo (Saif, 1981). Relativamente à nutrição azotada, factor geralmente limitante para as plantas no sapal, as micorrizas poderão ter algum papel na tomada de azoto inorgânico, embora a maioria do azoto se encontre na forma orgânica (Valiela & Teal, 1979; Cartaxana, 1999).

Em condições de alagamento os custos para a planta (hidratos de carbono) em manter a associação podem ser maiores que os benefícios nutricionais. Alguns autores têm sugerido que os fungos AM podem actuar como parasitas quando o solo está saturado durante longos períodos, e como mutualistas quando o solo fica mais seco (Liberta *et al*., 1983; Anderson *et al*., 1984). Johnson e colaboradores (1997) sugeriram que, em sistemas naturais, as respostas das plantas às micorrizas podem variar ao longo de um contínuo de respostas positivas a neutras e negativas, dependendo do estádio de desenvolvimento da planta e das condições ambientais. Ainda não são conhecidos dados que apoiem esta hipótese de um continuo entre parasitismo ou comensalismo e mutualismo nas AM dos sapais.

Outros possíveis benefícios das AM consistem no aumento da tolerância das plantas ao alagamento salino. Têm sido observado nalguns estudos benefícios da colonização AM em condições de salinidade para espécies vegetais de habitats salinos, essencialmente através da melhoria das relações hídricas e da nutrição (Rozema *et al*., 1986; Tsang & Maun, 1999). Em contraste, outros trabalhos não mostram qualquer benefício da presença de AM em condições salinas (Allen & Cunningham, 1983; Baker *et al*., 1995). Existem poucas evidências mostrando que as AM podem favorecer o crescimento de plantas em condições de solo alagado (Osundina, 1998; Miller & Sharitz, 2000). No entanto, não se conhecem trabalhos que tenham abordado a interacção dos factores alagamento e salinidade, que constituem as condições comuns nos sapais devido à acção das marés.

1.3. Objectivos

Na actualidade é amplamente reconhecida a importância ecológica das micorrizas, existindo uma preocupação crescente na necessidade de compreender a função ecológica dos fungos AM nos diferentes ecossistemas (van der Heijden & Sanders, 2002). O conhecimento das associações micorrízicas nos sapais, sistema complexo e com condições adversas, ainda é escasso. O conhecimento da ecologia dos fungos AM dos sapais, sua distribuição e carácter

adaptativo, e dos seus efeitos na sobrevivência e crescimento das plantas perante o alagamento salino é essencial para compreender a função que as AM desempenham nestes ecossistemas. Em particular, um amplo conhecimento das respostas associações micorrízicas será importante para prever o impacto na vegetação de sapal do aumento do nível da água do mar resultante das mudanças climáticas globais que se prevêem ir afectar consideravelmente os sapais (Donnelly & Bertness, 2001; Adam, 2002).

Este trabalho, tendo fundamentalmente como local de estudo o sapal de Pancas situado no estuário do Tejo, teve como principal objectivo contribuir para um maior conhecimento do significado ecológico das associações micorrízicas nos sapais. Tendo presente este objectivo geral, desenvolveram-se estudos subordinados aos seguintes objectivos específicos:

1. Caracterizar a ocorrência temporal e espacial das AM nos sapais, com vista a avaliar a sua relação com a identidade das espécies vegetais, a fenologia e a distribuição heterogénea da vegetação e dos factores edáficos determinada pela acção das marés;
2. Caracterizar a diversidade e os propágulos de fungos AM, de modo a avaliar o potencial de inóculo existente nos sapais;
3. Avaliar a tolerância dos fungos AM nativos às condições adversas dos sapais, nomeadamente à salinidade e ao alagamento;
4. Avaliar a infectividade dos fungos AM nativos (capacidade de infectar o sistema radicular) em plantas de sapal com condições de alagamento e salinidade;
5. Avaliar a contribuição dos fungos AM nativos na tolerância das plantas de sapal ao alagamento com água salina por acção da maré.

Os objectivos foram abordados em cinco artigos, que constituem os capítulos 2 a 6.

No Capítulo 2 foi caracterizada a ocorrência, a diversidade e a abundância, em termos temporais e espaciais, de fungos AM em plantas de sapal presentes em duas zonas do sapal sujeitas a diferentes regimes de alagamento, e consequentemente, a distintas características edáficas. Foi dada especial atenção à variação do nível de colonização das plantas por fungos AM e da densidade de um dos tipos principais de propágulos dos fungos AM, os esporos, e sua relação com características fenológicas e edáficas.

Atendendo à ocorrência conjunta de plantas que formam AM e de plantas que não formam qualquer associação do tipo micorrízica, e ao padrão de distribuição zonal da vegetação característico dos sapais, foi estudada no Capítulo 3 a relação entre o padrão de distribuição espacial dos propágulos esporos, a distribuição das plantas hospedeiras dos fungos AM nos

sapais e algumas propriedades edáficas. Como termo comparativo este estudo foi também realizado num tipo de ecossistema distinto com comunidades de plantas apresentando maior riqueza de associações micorrízicas.

No Capítulo 4 foi estudada a tolerância dos fungos AM nativos dos sapais às condições de stress presentes nestes ecossistemas (salinidade e alagamento), comparativamente com isolados de referência colhidos noutro tipo de ecossistema. Neste capítulo é também investigada a contribuição de diferentes tipos de propágulos de fungos AM para a colonização das plantas nativas do sapal.

O potencial dos fungos AM presentes nos sapais para iniciar e desenvolver a colonização em *A. tripolium* (planta hospedeira de fungos AM presente em ambas as zonas do sapal, menos e mais alagada) sob diferentes condições de salinidade e alagamento (cada factor individualmente e em interacção) foi estudado no Capítulo 5.

No Capítulo 6 foi investigada a influência dos fungos AM na tolerância das plantas de sapal ao alagamento com água salina pela acção da maré. Foram estudados parâmetros de crescimento de duas populações distintas de *A. tripolium*, com a intenção de verificar se os efeitos das AM são ou não dependentes da origem do hospedeiro.

No Capítulo 7 os principais resultados foram resumidos e discutidos no âmbito do significado ecológico das micorrizas nos sapais.

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CAPÍTULO 2

Temporal and spatial variation of arbuscular mycorrhizas in salt marsh plants of the Tagus estuary (Portugal)

Este capítulo constitui integralmente o seguinte artigo:

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Temporal and spatial variation of arbuscular mycorrhizas in salt marsh plants of the Tagus estuary (Portugal)

Luís M. Carvalho · Isabel Caçador · M. A. Martins-Loução

Luís M. Carvalho · M. A. Martins-Loução
Centro de Ecologia e Biologia Vegetal, Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal.
Isabel Caçador
Instituto de Oceanografia, Faculdade de Ciências de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

Abstract

The factors which may influence temporal and spatial variation in plant arbuscular mycorrhizal (AM) colonization and propagule occurrence were evaluated in a Portuguese salt marsh poor in plant diversity. Two distinct sites were studied: a more-flooded (low marsh) and a less-flooded zone (high marsh). AM root colonization, AM fungal spore number and inoculum potential, soil edaphic parameters and tidal flooding time periods were analysed. Levels of AM colonization were considerable in *Aster tripolium* and *Inula crithmoides* but very low in *Puccinellia maritima* and non-existent in *Spartina maritima*, *Halimione portulacoides*, *Arthrocnemum fruticosum* and *Arthrocnemum perenne*. Fungal diversity was very low, with *Glomus geosporum* dominant at both marsh zones. Colonization showed no spatial variation within marsh zones but temporal variation was observed in the high marsh, dependent on plant phenological phases. In the low marsh, no significantly seasonal variation was observed. Apparently plant phenological events were diluted by stressful conditions (e.g. flooding, salinity). Spore density was significantly different between marsh zones and showed temporal variation in both zones. This study showed that distribution of mycorrhizas in salt marsh is more dependent on host plant species than on environmental stresses.

Key words Arbuscular mycorrhizas · Salt marshes · Temporal and spatial variation · Flooding · Salinity

Introduction

Salt marshes are ecosystems where tidal flooding with seawater leads to a partial or total submergence of vegetation, high soil salinity and soil anoxia. Soil inundation creates anaerobic and chemically reduced conditions around plant roots, leading to oxygen deficiency and phytotoxin accumulation (Armstrong et al. 1991). As a result, plant growth and survival, species composition and zonation patterns are strongly influenced by flooding and the concentrations of salt and oxygen (Armstrong et al. 1985; Pennings and Callaway 1992). Generally, soil salinity, soil moisture and anaerobiosis decrease from the lower to the higher

zone of a salt marsh, creating a zonated pattern in the vegetation. Salt marsh plants exhibit biochemical, morphological and physiological adaptations to waterlogging and salinity (Armstrong et al. 1991; Naidoo et al. 1992). Microorganisms in the root zones, particularly arbuscular mycorrhizal fungi (AMF), may enhance ecological adaptation of these plants, including pioneer plant colonizers to salt marsh environments (Sengupta and Chaudhuri 1990; Khan and Belik 1995). Some evidence suggests that mycorrhizas improve plant tolerance to salinity (Jindal et al. 1993; Ruiz-Lozano et al. 1996), though other results show that mycorrhizal infection can be suppressed by high soil salinity (Pfeiffer and Bloss 1988; Juniper and Abbott 1993) and waterlogging (Harley and Smith 1983). Recently, Miller (1999) showed that flooding partially inhibits AM colonization of wetland grasses.

Despite the stressful environment, occurrence of arbuscular mycorrhizas has been reported in salt marsh plants in several countries, including the Netherlands (Rozema et al. 1986; Van Duin et al. 1990; Hildebrandt et al. 2001), Germany (Hildebrandt et al. 2001), Great Britain (Mason 1928; Read et al. 1976), France (Boullard 1958), United States (Cooke and Lefor 1990; Cooke et al. 1993; Hoefnagels et al. 1993; Brown and Bledsoe 1996), India (Sengupta and Chaudhuri 1990) and Pakistan (Khan 1974). No previous assessment has been made of Portuguese salt marshes.

In mediterranean-type salt marshes, the characteristic climate (wet winters and hot and dry summers) causes salinity to fluctuate seasonally and along a gradient (Callaway et al. 1990). These zonal differences in abiotic characteristics cause plant zonation that, associated with plant phenology, may influence spatial and temporal patterns of arbuscular mycorrhizas.

Few studies have tried to identify the principal factors responsible for spatial and temporal variation in AM colonization of salt marsh plants. Plant phenology events and abiotic stresses, such as flooding and salinity, have been suggested (Rozema et al. 1986; Cooke and Lefor 1990; Van Duin et al. 1990; Brown and Bledsoe 1996). Brown and Bledsoe (1996) suggested that AM colonization is influenced by salinity in the higher marsh zone and by oxygen availability levels due to tidal inundation in the more flooded zones (low marsh). However, in a controlled experiment with the salt marsh plant *Aster tripolium* Rozema et al. (1986) found a decrease in AM colonization with increased flooding but not with raised soil salinity.

This present study aimed to assess the AM status of plant species in a Portuguese salt marsh poor in plant diversity. The presence of AM infection in plant species posed, subsequently, two main questions: (1) did the incidence of AM colonization and spore density vary spatially according to different marsh zones with different tidal flooding regimes; (2) were AM colonization and spore density related to phenological events of plant species or to

edaphic conditions? To answer these questions, plant species present in lower and higher areas of the salt marsh were evaluated in order to assess response of mycorrhizas to biotic and abiotic variables. Temporal and spatial variation in salinity, soil moisture, soil organic matter, redox potential and tidal flooding periods was assessed and compared to patterns of mycorrhizal colonization and spore density.

Materials and methods

Study site

This work was performed in a marsh of the Tagus estuary, Portugal. This is one of the largest estuaries of the European Atlantic coast, covering an area of 300 km^2 at low tide and 340 km^2 at spring high tide (Caçador et al. 1996). The climate is Mediterranean, characterized by warm and dry summers and cold and wet winters with 600-700 mm mean annual precipitation. The tides are semi-diurnal with tidal ranges from less than 1 m (neap) to more than 4 m (spring). The salinity varies between 5‰ upstream and 36‰ near the river outfall (Lisbon) (Caçador 1994). The estuary is exposed to high pollution due to the inflow of urban, industrial and agricultural effluents. The salt marsh vegetation acts as a sink concentrating high levels of heavy metals in the rhizosphere sediments (Caçador 1994). About 20 km^2 of the estuary are occupied by salt marshes, with Pancas being one of the largest, located on the left bank (38º49´N 08º57´W). This salt marsh links with agricultural lands in the higher marsh zone and to extensive inter-tidal mudflat areas in the lower marsh zone. The soils are clay (97% particle size <63 μm) and characteristics in the lower and higher marsh zone, respectively, are: pH 6.0 and 6.3; 2 and 3 g kg^{-1} total N; 900 and 792 mg kg^{-1} total P; 7,476 and 2,498 mg kg^{-1} total Ca; 1,813 and 1,902 mg kg^{-1} total K; 2,369 and 2,658 mg kg^{-1} total Mg. The halophytic vegetation of this marsh presents very low plant diversity compared wth other northern European salt marshes. It also shows distinct and almost homogeneous plant species stand zones. The dominant species are *Spartina maritima* (Curtis) Fernald, *Arthrocnemum perenne* (Miller) Moss, *Arthrocnemum fruticosum* (L.) Moq., *Halimione portulacoides* (L.) Aellen and *Aster tripolium* L. The lower marsh zone (pioneer zone) of this salt marsh is regularly flooded with salt water and the higher marsh zone (more elevated site) is flooded by a channel system but only during high tides and high precipitation.

Two sampling areas of 300 m^2 were established, one in the lower marsh and another in the higher marsh. The plant species present were *Spartina maritima*, *Puccinellia maritima* (Huds.) Parl., *Halimione portulacoides, Arthrocnemum fruticosum* and *Aster tripolium* in the lower marsh sampling area and *Halimione portulacoides*, *Arthrocnemum fruticosum, Arthrocnemum perenne*, *Aster tripolium* and *Inula crithmoides* L. in the higher marsh sampling area.

Hours of tidal flooding

The tidal level above mean sea level required to flood each of the sampling areas was determined during some tides by means of stakes and on tide data published by the Hydrographical Institute. The mean time per day that vegetation was flooded at each sampling area was estimated using a model developed by Serôdio and Catarino (2000).

Plant root sampling

At each sampling area, plant roots were sampled at 2-month intervals between July 1996 and July 1997. For each survey, root systems and associated soil from three randomly selected plants of each species present in the sampling areas were carefully collected at low tide to a depth of approximately 20 cm. The samples were transported to the laboratory in plastic bags and stored at 4ºC until processed.

Soil sampling

Soil samples from *Aster tripolium* stands in both sampling areas were also collected on the root sampling days. At each marsh zone, four sediment cores (7 cm in diameter, 18 cm long) were collected. After first removing the uppermost 1 cm sediment layer, the cores were transported to the laboratory in plastic bags and stored at 4ºC until processed. In order to compare sediment redox potential between marsh zones, measurements were made in May and July 1997. After high tide, a platinum electrode was inserted into the rhizosphere sediment of *Aster tripolium* at a depth of 10 cm and the redox potential (Eh) was recorded after a stabilisation period of 2 min. Four measurements were made in each zone on each sampling date.

AM root colonization

After carefully rinsed with tap water, approximately 2 g (fresh weight) of roots was cleared and stained for analysis of colonization by AMF using a modified Phillips and Hayman (1970) procedure. The roots were cleared for 40-60 min (according to the species) in a 10% KOH solution at 90ºC, placed in 10% HCl solution for 10 min and then stained with glycerol-trypan blue solution (0.05%) at 90ºC for 20 min. The stained root samples were examined at x45-100 magnification and quantification of root colonization by AMF was estimated by the gridline intersection method (Giovannetti and Mosse 1980). When it was difficult to discriminate between mycorrhizal structures and other fungi, the root pieces were examined microscopically at x400 magnification.

AMF spore population

AMF spores were isolated by wet sieving followed by sucrose gradient centrifugation (Daniels and Skipper 1982). From each core sample of the *Aster tripolium* rhizosphere, 50 g of sediment was sieved. The fraction collected in the last sieve (53µm) was centrifuged in a 60% (w/v) sucrose solution for 2 min at 3,000 rpm. Spores were collected from the water-sucrose interface and poured through a sieve, rinsed with distilled water and transferred to a Petri dish. Spores were examined under a dissecting microscope and each morphotype quantified. Spore density was expressed as spore number per g of dry weight soil. Some spores of each morphotype were then prepared permanently for identification. Taxonomic identifications were made according to Banque Européenne des Glomales www.bio.ukc.ac.uk/beg.htm and Schenck and Pérez (1990).

Spore viability tests were performed to examine differences between marsh zones in samples isolated in July 1997. From each sample, 40 isolated spores were randomly collected, placed in iodonitrotetrazolium (INT) solution (1 mg ml^{-1}) and left at room temperature for 48 h (Walley and Germida 1995). Spores were checked for viability and the results expressed as viable percentage.

Soil analysis

Aliquots (10 g) of each soil core sample was heated at 80ºC for 48 h then weighed. Soil moisture content was calculated as percent oven-dry weight of soil. Organic matter content was determined in 2 g of dried soil without roots of each sample by loss of ignition (LOI) at 600ºC for 2 h (Otte 1991). Soil solution salinity was calculated in each soil sample. Aliquots

(2 g) of dried soil were ground, sieved and diluted with deionized water (1:5). After overnight shaking, the soil solution was filtered and its salinity determined with a hand-held refractometer.

AM inoculum potential

The total number of AMF propagules in the salt marsh sediment was estimated using a soil-dilution method, the "most probable number" (MPN) method (Porter, 1979). Sediments from the root zone of *Aster tripolium*, from higher and lower marsh zones, and from *Spartina maritima* from the lower marsh zone, collected in September 1996, were used. Each sediment sample was diluted with sterilized sand in a serial dilution of 4^0 to 4^{-6}. The control contained only sterilised sand. The mixture was placed in 120 ml containers and seedlings of sorghum were used as bait plants for AMF. Five replicates were used for each dilution level. The plants were grown in a greenhouse with photoperiod of 14 h, 26/20ºC (day/night air temperature) and a photosynthetic photon flux density of 200 μmol m^{-2} s^{-1} and watered as necessary. After 8 weeks, plant roots were collected and analysed for AM colonization as described above. The number of propagules was calculated according to Sieverding (1991).

Statistical analysis

Prior to statistical analysis, mycorrhizal colonization data were arcsin square root-transformed and data for spore number, LOI, soil salinity and number of tidal flooding hours of the vegetation over the last 14 days (number of days corresponding to a tidal cycle) were log-transformed (Zar 1984). Data on mycorrhizal colonization and spore densities were analysed by one-way analysis of variance (ANOVA) to test for differences between the two marsh zones and between sampling dates within each marsh zone. Significant results ($P < 0.05$) were analysed by Duncan's test. MPN data were expressed with 95% confidence limits. Pearson product-moment correlation analysis was used to relate AM colonization and spore density with soil moisture, LOI, soil salinity and number of tidal flooding hours of the vegetation in each sampling date of *Aster tripolium* roots or soil rhizosphere in each marsh zone separately. Statistica software (StatSoft, Tulsa, USA) was used for this statistical analysis.

Results

Climatic and edaphic analyses

In Pancas, lower marsh vegetation was much more flooded due to tides than higher marsh vegetation, as expected (Table 1). During June and December 1996 and May 1997, the higher marsh vegetation was not tidally flooded. Soil moisture, salinity and organic matter content (LOI) were higher in the lower than in the higher marsh zone (Table 1). Soil moisture is more influenced by tide than by seasonal precipitation. Most precipitation in the study period occurred in the winter months of December and January (data not shown). While the lower marsh zone showed a marked seasonal variation of salinity, the higher marsh zone did not. The lower marsh zone exhibited an average redox potential much lower than the higher marsh zone (Table 1).

Table 1 Time of tidal flooding of the vegetation, obtained from monthly values through the sampling periods, and edaphic characteristics and spore density in soils collected from *Aster tripolium* stands on seven sampling dates, except for redox potential which is from two sampling dates. The values are means with ranges in parentheses (*AT Aster tripolium*, *MPN* most probable number with confidence limits of 95%, *SM Spartina maritima*)

Variable	Low marsh	High marsh
Tidal flooding of the vegetation (min day^{-1})	94 (53-124)	9 (0-21)
Moisture (%)	77 (74-89)	45 (27-57)
Soil organic matter content (LOI) (%)	11.9 (10.5-13.1)	13.0 (9.2-18.1)
Salinity (‰)	18 (3-31)	12 (5-18)
Redox potential (mV)	194 (100-289)	402 (381-423)
Spore density (g^{-1} dry wt.)	6 (2-14)	13 (5-34)
MPN (propagules g^{-1} dry wt.) (AT)	0.75 (0.35-1.60)	1.74 (0.81-3.72)
MPN (propagules g^{-1} dry wt.) (SM)	0.17 (0.08-0.35)	

AM root colonization

AMF were only present in root samples of three salt marsh plant species, *Aster tripolium*, *Inula crithmoides* and *Puccinellia maritima*, in both marsh zones (Table 2). The level of AM colonization in *Inula crithmoides* was similar to that in *Aster tripolium*, while *Puccinellia maritima* displayed little or no colonization (1% on average). Mycorrhizal colonization was not observed in roots of *Spartina maritima*, *Halimione portulacoides*, *Arthrocnemum fruticosum* and *Arthrocnemum perenne* from any marsh zone on any sampling date (Table 2). In *Aster tripolium* and *Inula crithmoides*, both arbuscules and vesicles were always observed. Arbuscules were never observed in *Puccinellia maritima* (Table 2). *Puccinellia maritima* was

only colonized by AMF on two sampling dates, January and March, but with very low values, 5% and 2%, respectively (Fig. 1).

Table 2 Range of root length colonization by AMF (%), with sampling every 2 months from July 1996 to July 1997 in all plant species present in the lower and higher marsh zones. *n*=21, except for *Inula crithmoides* where *n*=18 (*A* arbuscules, *V* vesicles)

Zone	Plant Species	Plant Family	Root length colonized (%)	Mycorrhizal structures
Lower	*Aster tripolium*	Asteraceae	1-56	VA
	Puccinellia maritima	Poaceae	0-6	V
	Spartina maritima	Poaceae	0	Absent
	Halimione portulacoides	Chenopodiaceae	0	Absent
	Arthrocnemum fruticosum	Chenopodiaceae	0	Absent
Higher	*Aster tripolium*	Asteraceae	3-63	VA
	Inula crithmoides	Asteraceae	6-62	VA
	Halimione portulacoides	Chenopodiaceae	0	Absent
	Arthrocnemum fruticosum	Chenopodiaceae	0	Absent
	Arthrocnemum perenne	Chenopodiaceae	0	Absent

Aster tripolium was the only AMF-colonized plant species present in both marsh zones (Table 2). One-way analysis of variance was performed on colonization data to examine temporal and/or spatial patterns (Table 3). No significant variation in colonization as a function of salt marsh zone sampled was observed. On the contrary, significant variation in colonization was found in the higher marsh zone for both *Aster tripolium* and *Inula crithmoides* (Fig. 1 and Table 3). In this zone, colonization was higher in summer and autumn than in winter and spring sampled months (Fig. 1); this was particularly evident for *Aster tripolium*. In the lower marsh zone, colonization of *Aster tripolium* did not differ significantly between sampling dates (Fig. 1 and Table 3).

Table 3 Summary of one-way analysis of variance of the effect of marsh zone (lower and higher) and sampling date on root AM colonization and soil spore density for *Aster tripolium* and of the effect of sampling date on root AM colonization for *Inula crithmoides*

		d.f.	Colonization *F* value	Spore density *F* value
Marsh zones	*Aster tripolium*	1	0.89	17.64***
Sampling date	*Aster tripolium* (lower marsh)	6	1.51	4.79**
	Aster tripolium (higher marsh)	6	3.70*	11.72***
	Inula crithmoides (higher marsh)	5	6.54**	

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

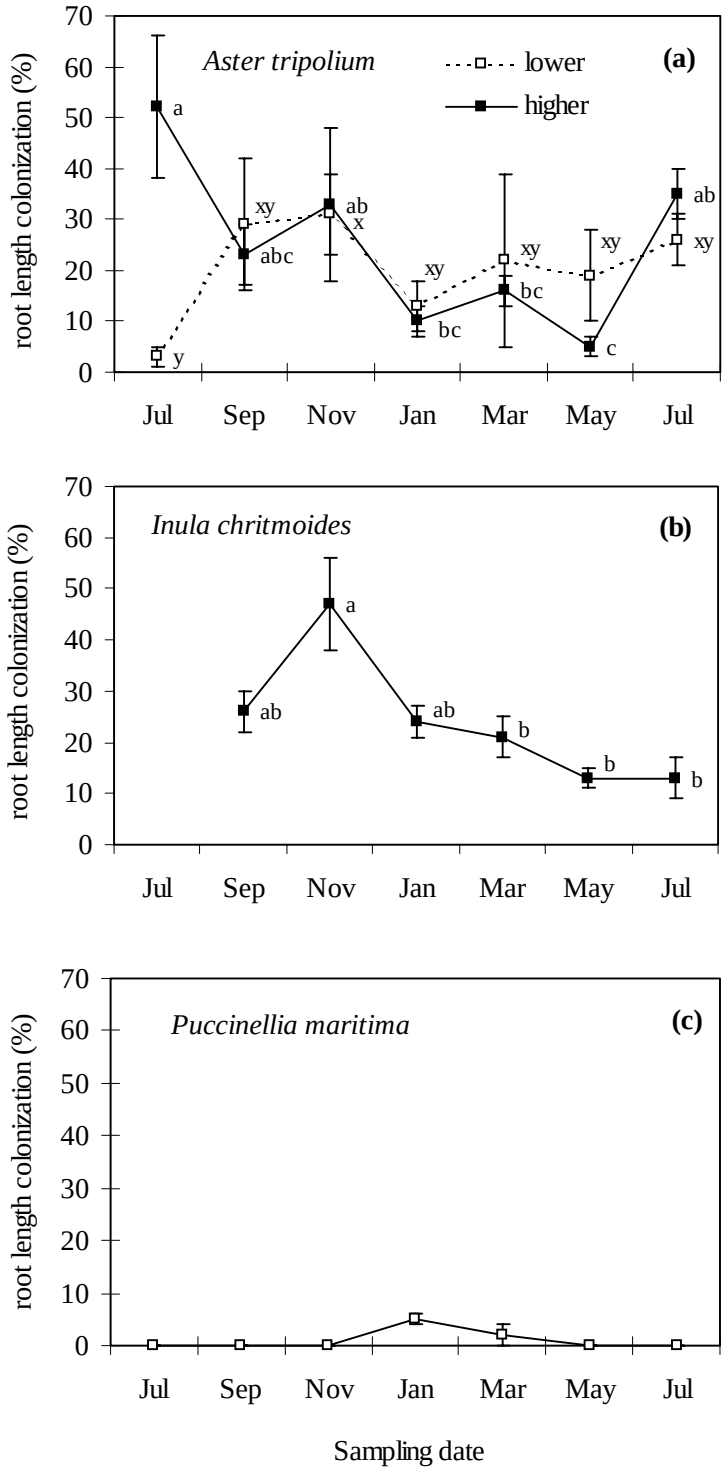

Fig. 1 Temporal and spatial variation of root length colonization by arbuscular mycorrhizal (AM) fungi on **a** *Aster tripolium* plants of the lower and higher marshes zones, **b** *Inula crithmoides* plants of the higher marsh zone and **c** *Puccinellia maritima* plants of the lower marsh zone. Values are means of three replicates per month and marsh zone ± SE. In each graph and within each marsh zone, values followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

AMF spore population

The AMF spores found in the soil samples belonged exclusively to *Glomus* species and all of the species were present at both salt marsh zones. Spores of *G. geosporum* (Nicol. and Gerd.) Walker were the most common, accounting for more than 84% of the total AMF spore population. *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe was also present, along with two other unidentified *Glomus* species. Spore density varied both spatially and temporally (Tables 1 and 3). The patterns of temporal variation in spore density in the two salt marsh zones were similar (Fig. 2). Spores from both marsh zones were highly viable: 67% (±3 SE) in the higher marsh zone and 71% (±3 SE) in the lower marsh zone.

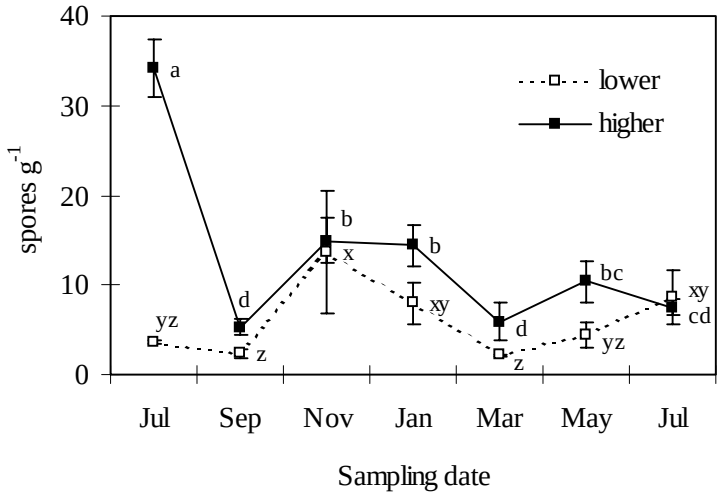

Fig. 2 Temporal and spatial variation on spore density (per g dry soil) in rhizosphere soil of *Aster tripolium* from the lower and higher marshes zones. Values are means of four replicates per month and marsh zone ± SE. Within each marsh zone, values followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

AM inoculum potential

The results of mycorrhizal infectivity determined by a soil-dilution method showed differences between soils tested. The soil collected from *Aster tripolium* stands was significantly more infective than the soil from *Spartina maritima* (Table 1) and that collected from the higher marsh zone had more infective propagules than that from the lower marsh zone (Table 1). The *Spartina maritima* soil had some infective propagules, although roots were never colonized (Table 2).

Effect of edaphic conditions

AM colonization and spore density were not related to soil moisture, LOI and salinity, except in the higher marsh zone, where AM colonization was positively correlated with soil salinity (Table 4). AM colonization in the low marsh was significantly positively correlated with the number of tidal flooding hours of the vegetation over the previous 14 days before sampling took place (Table 4).

Table 4 Pearson product-moment correlation coefficients at each sampling date between % of AM colonization and spore density in *Aster tripolium* samples of each marsh zone and soil moisture, soil organic matter content (LOI), soil salinity and number of tidal flooding hours of the vegetation over the last 14 days (number of days corresponding to a tidal cycle). Coefficients were calculated from means obtained for each sampling date (n=7)

		Moisture	LOI	Salinity	Flooding hours
Low marsh	% AM	-0.316	0.076	-0.007	0.776*
	Spore density	-0.119	0.590	-0.341	0.130
High marsh	% AM	-0.199	-0.259	0.820*	0.123
	Spore density	0.430	0.299	0.183	-0.219

* P<0.05

Discussion

This study showed that arbuscular mycorrhizas were present in the Pancas salt marsh of the tagus estuary and that colonization and propagules occurred in higher and lower marsh zones. However, not all plant species were colonized and only the species belonging to the Asteraceae family, *Aster tripolium* and *Inula crithmoides*, displayed high levels of colonization. The presence of a considerable level of colonization in *Aster tripolium* roots had also been reported in salt marshes by Rozema et al. (1986), Van Duin et al. (1989) and Hildebrandt et al. (2001). AM colonization in *Puccinellia maritima* occurred at very low levels and only in winter months, a period of slow plant growth. These data, together with the absence of arbuscules, suggest that this plant species is not functionally mycorrhizal.

All the plant species present in Pancas that were not colonized belong to the Chenopodiaceae family, with the exception of *Spartina maritima*, a member of Poaceae. Chenopodiaceae is usually referred to as non-mycorrhizal (Harley and Smith 1983). However, AM has also been found in some plants of this family, not only in other salt marshes but also in other types of ecosystems (e.g. Rozema et al. 1986; Van Duin et al. 1989; Sengupta and

Chaudhuri 1990; Johnson-Green et al. 1995; Barrow et al. 1997; Hildebrandt et al. 2001). *S. maritima* may be referred to as a non-mycorrhizal species together with *S. alterniflora* and *S. anglica* (Rozema et al. 1986; Hildebrandt et al. 2001). In contrast, mycorrhizas have been observed in the *Spartina* species *S. patens* (Cooke and Lefor 1990; Hoefnagels et al. 1993) and *S. cynosuroides* (Hoefnagels et al. 1993).

AMF species diversity was very low, accompanying the low plant diversity of this salt marsh ecosystem (Caçador 1994). Only *Glomus* species were found. *G. mosseae*, observed in Pancas salt marsh, has also been reported in other marshes (Sengupta and Chaudhuri 1990; Brown and Bledsoe 1996). Interestingly, the most abundant *Glomus* species was *G. geosporum*, as was previously found in Baltic salt marshes (Hildebrandt et al. 2001).

The evaluation of plant species present in two different sites in the marsh showed that the occurrence of AM colonization did not depend on the position within the salt marsh nor, consequently, on the tidal flooding regimes which create different levels of anoxia around the roots, nor on salt gradients. This suggests that AM fungal distribution does not coincide with the zonation pattern of vegetation. The absence of any significant negative correlation between AM and salinity, moisture and tidal inundation of the vegetation seems to suggest that AMF can tolerate the flooding and salinity levels in the Pancas salt marsh.

The evaluation of biotic and abiotic factors responsible for AM seasonal patterns has shown that plant phenology is related to AM colonization in *Aster tripolium* and *Inula crithmoides* and spore density, more clearly in the higher marsh zone. The highest levels of colonization corresponded to the period of the highest plant growth and the flowering period in both species, summer and autumn, respectively, in agreement with the results of Van Duin et al. (1989). Similar seasonal patterns in spore density have been observed in aquatic plants (Khan 1974) but not for the halophyte *Jaumea carnosa* (Brown and Bledsoe 1996). These differences may be related either to the different behaviour of each AM fungal species, even in similar ecosystems (Klironomos et al. 1993), or to the influence of different environmental conditions.

AM colonization and spore distribution may also be influenced by tides, since *S. maritima*, a non-mycorrhizal plant which occupies a frontal isolated zone in the marsh, showed a reasonable number of propagules and a significantly positive correlation between AM colonization and tidal flooding hours was observed.

In the lower marsh zone, the lack of seasonality and the small variation in the AM colonization in *Aster tripolium* suggest that either other factors influence fungal development more than plant phenology or other factors dilute its influence. Brown and Bledsoe (1996)

also did not find significant seasonal variation in colonization in the most waterlogged zone of a salt marsh.

The presence of AM colonization in few plant species and in species that have a limited cover in all salt marsh vegetation suggests that mycorrhizas are of limited relevance for plant communities of the Tagus estuary salt marshes. However, since *Aster tripolium* is a species which occurs in less consolidated sediments and in more flooded areas, the presence of AM in this plant suggests that mycorrhizas have an important role during plant establishment and community development, at least, of this salt marsh plant species. Compared with observations in other salt marsh ecosystems (e.g. Rozema et al. 1986; Van Duin et al. 1990; Hildebrandt et al. 2001), it may be asked why so few plant species are mycorrhizal in this Portuguese salt marsh. Hypothesis that can be formulated for further study include the existence of high levels of phosphorous and the amount of clay in the soil (more than 90 %); iii) the presence of few potential mycorrhizal plant families. In these harsh environments, the interaction between host plant species and abiotic factors are so complex that it is difficult to explain AM colonization patterns in terms of general factors common to all salt marshes.

The results of this study emphasize that host plant rather than environmental stress factors are responsible for AMF distribution. Like the plant species, AMF may have developed adaptive strategies to tolerate this stressful environment. The results of this study rise questions important to our understanding of the role of mycorrhizas in the ecology of salt marshes.

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CAPÍTULO 3

Spatial variability of arbuscular mycorrhizal fungal spores in two natural plant communities

Este capítulo constitui integralmente o seguinte artigo:

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Spatial variability of arbuscular mycorrhizal fungal spores in two natural plant communities

Luís M. Carvalho[1], Patrícia M. Correia[1], Ronald J. Ryel[2] and M. Amélia Martins-Loução[1]

[1]Centro de Ecologia e Biologia Vegetal, Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal.
[2]Department of Forest, Range and Wildlife Sciences and the Ecology Center, Utah State University, Logan, Utah 84322-5230, USA.

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Abstract

Geostatistical techniques were used to assess the spatial patterns of spores of arbuscular mycorrhizal fungi (AMF) in soils from two contrasting plant communities: a salt marsh containing only arbuscular mycorrhizal and non-mycorrhizal plants in a distinct clumped distribution pattern and a maquis with different types of mycorrhiza where most plants were relatively randomly distributed. Also evaluated was the relationship between the spatial distribution of spores and AM plant distribution and soil properties. A nested sampling scheme was applied in both sites with sample cores taken from nested grids. Spores of AMF and soil characteristics (organic matter and moisture) were quantified in each core, and core sample location was related to plant location. Semivariograms for spore density indicated strong spatial autocorrelation and a patchy distribution within both sites for all AM fungal genera found. However, the patch size differed between the two plant communities and AM fungal genera. In the salt marsh, AM fungal spore distribution was correlated with distance to AM plants and projected stand area of AM plants. On the other hand, in maquis spatial AM fungal spore distribution was correlated with organic matter. These results suggest that spore distribution of AMF varied between the two plant communities according to plant distribution and soil properties.

Introduction

Spatial heterogeneity is a general feature of soil properties (e.g., Farley and Fitter, 1999; Jackson and Caldwell, 1993a,b; Ryel et al., 1996), vegetation structure (e.g., Lavorel et al., 1991) and diversity of soil microorganisms (e.g., Klironomos et al., 1999) in natural ecosystems. Several studies have documented that spores of arbuscular mycorrhizal fungi (AMF) are in aggregated distribution (e.g., Anderson et al., 1983; Friese and Koske, 1991; Klironomos et al., 1999). Moreover, there is growing evidence that the diversity and distribution of AMF is related to plant community structure and ecosystem function (van der Heijden and Sanders, 2002).

Spores of AMF are the major form of propagules that can be identified accurately to species. Consequently, they are very important in determining AM fungal species distribution (Smith and Read, 1997). In some situations, AM fungal spore abundance has been shown to be related to variation in moisture (Anderson et al., 1983), organic mater (Klironomos et al., 1993), pH (Porter et al., 1987) and temperature (Koske, 1987). However, these relationships do not always hold. For instance, Anderson et al. (1984) did not observe a relationship with soil organic matter and pH. Friese and Koske (1991) in a small spatial scale study also did not find any correlation between AM fungal spore density and root location.

There is evidence suggesting that AM fungal composition has the potential to affect the structure and functioning of plant communities through their effects on plant growth, survivorship, competition and diversity (Francis and Read, 1994; Sanders and Koide, 1994; van der Heijden et al., 1998). Conversely, the composition of plant communities may affect the structure of AM fungal communities through differential survival and reproduction (e.g., sporulation rates) of AMF (Eom et al., 2000; Johnson et al., 1992; Sanders and Fitter, 1992). Thus, it is possible to suggest that there might be a relationship between AM fungal spores spatial distribution and plant community structure, particularly with spatial distribution of AM hosts. Terrestrial ecosystems differ greatly in the mycorrhizal status of the vegetation, ranging from plant communities with only non-mycorrhizal plant species or composed of these non-host species with plants belonging to one type of mycorrhizal association, to communities that contains plant species that form different mycorrhizal types. Furthermore, the diversity and abundance of the plant species that form AM associations can also differ among plant communities.

We hypothesized that the strength of the relationship between AM fungal spatial distribution and AM plant distribution may vary among plant communities in relation to their structure (composition of mycorrhizal types and spatial pattern of plant species). Anderson et al. (1983, 1984) and Johnson et al. (1991) found that AM fungal spore density can be related to plant cover. Klironomos et al. (1999), in a spatial distribution study, found that spore abundance of some AM fungal taxa was related to shrub location. However, in previous efforts comparative studies were not conducted among sites with differences in plant community structure. Thus, it is still unclear if soil properties or plant variables are most important in explaining the spatial distribution of AM fungal spores.

In this work, we assess the importance of plant spatial distributions and soil properties on the distribution of AM fungal spores in two plant communities of dissimilar structure. The approach used sampling and statistical procedures that permitted evaluation of AM fungal

spore distributions across many plant communities. The two plant communities chosen were a salt marsh and an evergreen sclerophyllous shrub community (maquis). The salt marsh has low plant diversity and is composed only of non-host and a few AM host plant species (Carvalho et al., 2001). The vegetation is spatially structured in clusters with distinct zones composed of nearly monospecific stands. Therefore, distinct zones of AM plants can be found surrounded by non-host plants. In maquis the organization of the vegetation has a higher complexity than in the salt marsh. Maquis has much higher plant species diversity and contains diverse types of mycorrhizas (Puppi and Tartaglini, 1991). The complexity of this community is increased by the presence of different layers (trees, shrubs and herbaceous). Most plants in this type of Mediterranean ecosystems are relatively randomly distributed (Keeley, 1992), although a few species may form clusters, frequently with the co-occurrence of individuals of others species inside the clusters. As a result AM plants intermingle with plants hosting other mycorrhizal types.

The specific goals of this study were: (i) to quantify the spatial variability in numbers of AMF spores, and (ii) to evaluate the relative importance of plant distribution and soil properties on the spatial distribution of AM fungal spores. To accomplish these goals, nested sampling schemes were used in the two chosen plant communities. Geostatistical techniques and correlation analysis were used to assess the influence of plant distribution and edaphic conditions on spore spatial distribution. Semivariance was used to express the degree of relationship between points on a surface (Burrough and McDonnell, 1998) and quantify the scale of autocorrelation (Rossi et al., 1992). These geostatistical procedures have been employed to quantify the extent of patches and variability of soil nutrients (e.g., Jackson and Caldwell, 1993a,b; Robertson et al., 1988; Ryel et al., 1996), and have also recently been used to describe spatial patterns in mycorrhizal fungi (Boerner et al., 1996; Klironomos et al., 1999). When combined with correlations analysis, soil and plant factors affecting the spatial patterns of AMF can be assessed.

Materials and methods

Study sites

The two study sites were located in Portugal: the salt marsh was situated at Pancas in the Tagus estuary (38º49’ N 08º57’ W) and the maquis was located in Arrábida Natural Park

(38º27´ N 09º02' W). In both sites the climate is Mediterranean, characterized by warm and dry summers and cool moist winters.

In the Tagus estuary, the tides have a semi-diurnal regime and about 20 km^2 are occupied by salt marshes, with Pancas being one of the largest. Soil in the Pancas salt marsh is predominantly clay with an average salinity of 15‰ and pH 6.2 (Caçador, 1994; Carvalho et al., 2001). The soil contains in average 2.5 g kg^{-1} total N and 846 μg g^{-1} total P. Low plant diversity and discrete, generally monospecific plant stands characterize the Pancas salt marsh community regularly flooded by seawater (Caçador, 1994). The main ecological factors constraining plant development are flooding and salinity. *Spartina maritima* (Curtis) Fernald occupies the riverside edge of the low marsh and is also present as islands of pure stands on intertidal mudflat areas. Distinct stands of *Arthrocnemum fruticosum* (L.) Moq., *Halimione portulacoides* (L.) Aellen and *Aster tripolium* L. are found more inland in the low marsh. *Puccinellia maritima* (Huds) Parl. is occasionally found in this marsh zone. The high marsh, the less flooded zone, is dominated by *Arthrocnemum perenne* (Miller) Moss. In this marsh zone, *A. fruticosum* and *H. portulacoides* stands are also common whereas *A. tripolium* and *Inula crithmoides* L. occasionally appear.

A general description of Arrábida is given in Catarino et al. (1982) and Clemente et al. (1996). The maquis is a dense scrub, characterized by the dominance of woody shrubs with evergreen sclerophyllous leaves and drought-deciduous shrubs. An overstory of small trees is sometimes present as well as an understory of annuals and herbaceous perennials. The main ecological factors constraining plant development are summer drought and nutrient availability (Correia, 1988). In the study site, the soil is clay-loam with a pH 7.1 and contains in average 0.25 g kg^{-1} total N and 66 μg g^{-1} total P. The current vegetation was established by resprouting and seed germination after a fire in 1986. At the time of the study, the vegetation was a shrubland ca. 1.5 m tall dominated by *Cistus monspeliensis* L., *Rhamnus lyciodes* (L.) Jahandiez & Maire, *Phillyrea angustifolia* L., *Quercus coccifera* L. and *Rosmarinus officinalis* L.. *Olea europaea* L. var. *sylvestris* Brot. and *Ceratonia siliqua* L. developed into small trees but the two species represented only a small percentage of plant cover. In the understory, *Phagnalon saxatile* (L.) Cass was a dominant species; several species of annual herbs and geophytes were also important components of the vegetation of this layer. Among plant species present in the study site, most were distributed at random; few had an aggregated distribution (e.g., *C. monspeliensis* and *Q. coccifera*) but not distinctly monospecific (A. Clemente, pers. comm.).

Sampling design

In June 1997, a 5 × 5 m experimental plot was established at each site at a randomly selected location within representative vegetation. Sampling followed a nested design. In each 5 × 5 m experimental plot ('macrogrid') 1-metre grids were marked. Samples were collected in the centre of 1 × 1 m cells. Within each 'macrogrids' one 1 × 1 m 'minigrid' was randomly selected. Grid lines were established at 0.2 m intervals and samples were collected from the centre of each 0.2 × 0.2 m cell. Within the 'minigrid' a randomly located 0.2 × 0.2 m 'microgrid' was established with samples taken at a finer scale of 0.05 m between adjacent points. There were 66 total samples taken in each site.

Core samples of 0.05 m diameter and 0.15 m depth were taken after removing the litter layer in the maquis and the uppermost 1-cm sediment layer in the salt marsh. Soil samples were taken to laboratory in plastic bags and stored at 4 ºC until processed. Plant diversity and their mycorrhizal status were recorded, percentage plant cover was estimated according to Braun-Blanquet cover classes and the projected area was mapped for each plant species. The mycorrhizal condition of the species was previously described in the salt marsh by Carvalho et al. (2001) and in maquis by the authors (unpublished data) or predicted from literature data (Puppi and Tartaglini, 1991). Distances of core samples to the closest AM plants were also measured. In salt marsh due to the clumped distribution of the vegetation, distance to the edge of the closest AM plant stands was considered instead of distance to individual plants.

Sample analysis

Spores of AMF were extracted from 30 g of each soil core sample by wet-sieving followed by sucrose gradient centrifugation (Daniels and Skipper, 1982). Water was added to 30 g of soil per sample and the solution passed over through a sequence of sieves (2000, 600 and 53 μm). The fraction collected in the last sieve (53 μm) was centrifuged in a 60% (w/v) sucrose solution for 2 min at 3000 rpm. Spores were collected from the water-sucrose interface, poured through a sieve, rinsed with distilled water and quantified under a dissecting microscope at ×45 magnification. Spores were counted at genus level and permanent slides of some selected spores from different samples were made and examined at ×400-1000 magnification. Spores were identified at least to a genus level according to the *Banque Européenne des Glomales*[1] and Schenck and Pérez (1990). Spore density was expressed as

[1] URL: http://www.ukc.ac.uk/bio/beg/

number of spores per 100 g dry soil. Gravimetric soil moisture content was calculated for each core sample as percent oven-dry weight of soil by drying at 80 ºC for 48 h. Soil organic matter content was calculated for each core sample using loss on ignition (LOI) at 600 ºC for 2 h (Otte, 1991).

Statistical analysis

Geostatistical variograms stratify calculated variances by the distance (or lag) between pairs of points (Isaaks and Srivastava, 1989). The variance of closer samples is more similar than of samples further apart (spatial autocorrelation). Variograms were calculated with GS+ program (Gamma Design Software, Plainwell, MI), using a minimum pair distance of 0.05 m and a maximum of approximately 4 m. Analyses were conducted using Isotropic Semivariation (assumes no directional difference in semivariance), because there was no evidence of anisotropy in the data. The data from spore densities were ln(x+1) transformed prior to analysis to improve normality, while the soil moisture and organic matter data were untransformed. The Spherical Model (Isaaks and Srivastava, 1989) showed the best fit to all the variograms. The Spherical Model is a modified quadratic function that assumes that sample points will not be autocorrelated beyond some distance; points were assumed not to be autocorrelated when the semivariance was equal to the sample variance (Ryel et al., 1996). The Spatial Dependence ($C/(C+C_0)\times100$), relates the Structural Variance (C) with Total Variance. The Nugget Variance (C_0) is the value of the semivariogram at extremely small distances (y-intercept of curve); this variance is not zero because there are several factors (such as sampling error) that causes this displacement at the origin of the semivariogram. The Sill ($C+C_0$) is the maximum value (plateau of semivariogram), representing the distance between points above which autocorrelation no longer exists. Coefficient of variation for samples in the large grid (5×5 m) was used as a measure of the magnitude of the variability among patches.

Correlations were calculated for each site to relate spore density with soil moisture, organic matter and AM plant location. All spatial relationships between variables were made in the 5×5 m grids. Plant location variables used were: projected area of the closest AM plants (maquis) or AM stands (salt marsh) and distance between sample core and the closest AM plants (maquis) or AM stands (salt marsh). Spearman rank-correlation coefficients were used due to skewed (non-normal) distributions of spore densities and plant variables.

Results

The two plant communities differed in plant diversity, plant mycorrhizal status, soil properties and spore number of AMF (Tables 1 and 2). In the maquis plot, several plant species belong to two different mycorrhizal types, AM and ectomycorrhiza, while in the salt marsh only AM occurred (Table 1). In maquis vegetation, AM plant diversity was higher than ectomycorrhizal plant diversity, although both plant covers were similar (data not shown). The co-occurrence of mycorrhizal host species with non-hosts species was only found in salt marsh. In the maquis sampling plot, only *Cistus monspeliensis* had a clustered distribution. Although this species establishes AM in the first stages of development and ectomycorrhiza as an adult plant, only fully developed *Cistus* were present in the study plot. Therefore, none of the AM plants occurred in a clustered distribution. Soil organic matter and moisture were much higher in salt marsh than in maquis (Table 2).

Table 1. Plant species composition in sampling plots from each site (salt marsh and maquis) and their mycorrhizal status (NM, nonmycorrhizal; AM, arbuscular mycorrhizal; ECM, ectomycorrhizal; AM/ECM, arbuscular mycorrhizal in young plants and ECM in mature plants)

Site	Plant species	Mycorrhizal status
Salt marsh	*Arthrocnemum fruticosum* (L.) Moq	NM
	Aster tripolium L.	AM
	Halimione portulacoides (L.) Aellen	NM
Maquis	*Anagallis arvensis* L.	AM
	Centaurium erythraea Rafn	AM
	Ceratonia siliqua L.	AM
	Cistus monspeliensis L.	AM / ECM
	Jasminum fruticans L.	AM
	Lavandula luisieri Rivas-Martínez	AM
	Olea europaea L. var. *sylvestris* Brot.	AM
	Phagnalon saxatile (L.) Cass.	AM
	Phillyrea angustifolia L.	AM
	Quercus coccifera L.	ECM
	Rhamnus lycioides (L.) Jahandiez & Maire	AM
	Rosmarinus officinallis L.	AM
	Rubia peregrina L.	AM / ECM

In the salt marsh only spores of the genus *Glomus* were found in the grid (Table 2). *Glomus geosporum* (Nicol. & Gerd.) Walker was the dominant species comprising almost 85% of the total. *Glomus mosseae* (Nicol. & Gerd.) Gerd. & Trappe and two other unidentified *Glomus* species were also found. In the maquis, spores of three different AMF genera were found: *Acaulospora*, *Scutellospora*, and *Glomus* (Table 2). *Glomus* spores were

the most common accounting for 75% of the total. The AMF species identified were *Glomus mosseae*, *Glomus geosporum* and *Scutellospora calospora* (Nicol. & Gerd.) Walker & Sanders. In addition, other unidentified species were found in the maquis sampling plot. Spore density was four times higher in the salt marsh than in the maquis (Table 2). Spores collected from maquis appeared much more damaged (empty or parasitized) than spores from salt marsh.

Table 2. Spore densities of AM fungal genera, organic matter and soil moisture for the samples collected on the 5 × 5 m 'macrogrid' in a salt marsh and from maquis vegetation. Spore density is expressed as spores per 100 g soil dry weight (n = 25)

Site	Variable	Mean	S.D.	C.V. (%)
Salt marsh	*Glomus* spore number	993	726	73.1
	Organic matter (%)	10.79	1.4	13.0
	Soil moisture (%)	72.80	4.2	5.8
Maquis	*Acaulospora* spore number	41	25	61.0
	Glomus spore number	181	135	74.2
	Scutellospora spore number	20	19	95.0
	Total spore number	242	155	64.0
	Organic matter (%)	3.8	2.0	52.6
	Soil moisture (%)	6.6	3.3	50.0

Table 3. Parameters for spherical model for semivariograms shown in Figures 1 and 2. Nugget Variance (C_0) is the value where the model line crosses the y-axis and represents the variance at very close distances (0.05 m). The Sill value ($C+C_0$) is the semivariance where there is no longer autocorrelation (each graph becomes a plateau) and A_0 is the corresponding distance between sample points beyond which there is no autocorrelation. Spatial dependence ($C/(C+C_0)$) is the ratio of structural variance to total variance

Site	Variable	Nugget (C_0)	Sill ($C+C_0$)	Range (A_0)	Spatial dependence (%)
Salt marsh	*Glomus*	0.010	3.555	6.55	99.7
	Organic matter	0.001	1.110	0.40	99.9
	Soil moisture	0.001	1.047	0.36	99.9
Maquis	*Acaulospora*	0.433	1.196	3.01	63.8
	Glomus	0.122	1.268	1.55	90.4
	Scutellospora	0.001	0.988	0.54	99.9
	Total spores	0.004	1.212	1.05	99.7
	Organic matter	0.001	0.160	0.90	99.9
	Soil moisture	0.001	1.146	0.98	99.9

In both sites, spores of AMF had an aggregated spatial distribution (Figures 1 and 2). Spatial dependence was very high for all spore isolates (except for *Acaulospora* spores in maquis) and soil properties, indicating strong autocorrelation within patches (Table 3). In maquis, for *Scutellospora* spores, total spores, organic matter and soil moisture, patches were

relatively small and well defined as reflected by high spatial dependence and autocorrelation between samples < 1 m (Table 3; Figure 2). Beyond this scale, each variogram was essentially flat, indicating the variables were no longer spatially correlated. For densities of *Acaulospora* and *Glomus* spores, the region of autocorrelation was greater than 1 m. In the salt marsh, all variables (*Glomus* spores and soil parameters) had high spatial dependence (Table 3; Figure 1). Soil parameters had a very small range of autocorrelation ($A_0 \leq 0.4$ m). However, *Glomus* spores had a much greater range of autocorrelation, estimated at 6.5 m. The nugget variances were low for all variables except for *Acaulospora* spores in maquis (Table 3; Figures 1 and 2). Low nugget variances indicate a higher internal uniformity compared to variability across the largest sample grid. The high nugget variance of *Acaulospora* spores indicated greater small scale heterogeneity than observed for other variables.

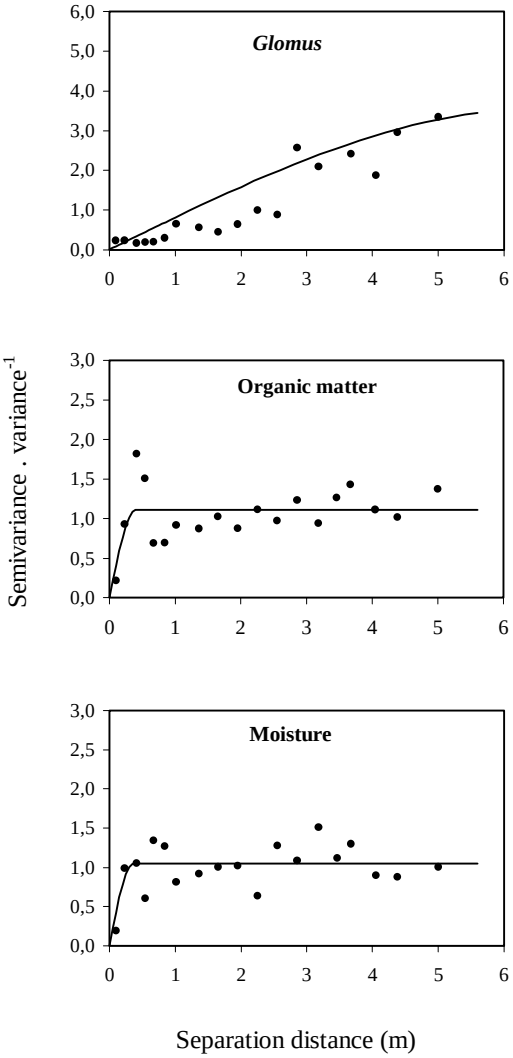

Figure 1. Semivariograms for *Glomus* spore density, soil organic matter and soil moisture in a salt marsh. Standardized semivariance was calculated by dividing semivariance by sample variance. Sample values were ln-transformed prior to semivariance and variance calculation. See Table 3 for parameter descriptions and values.

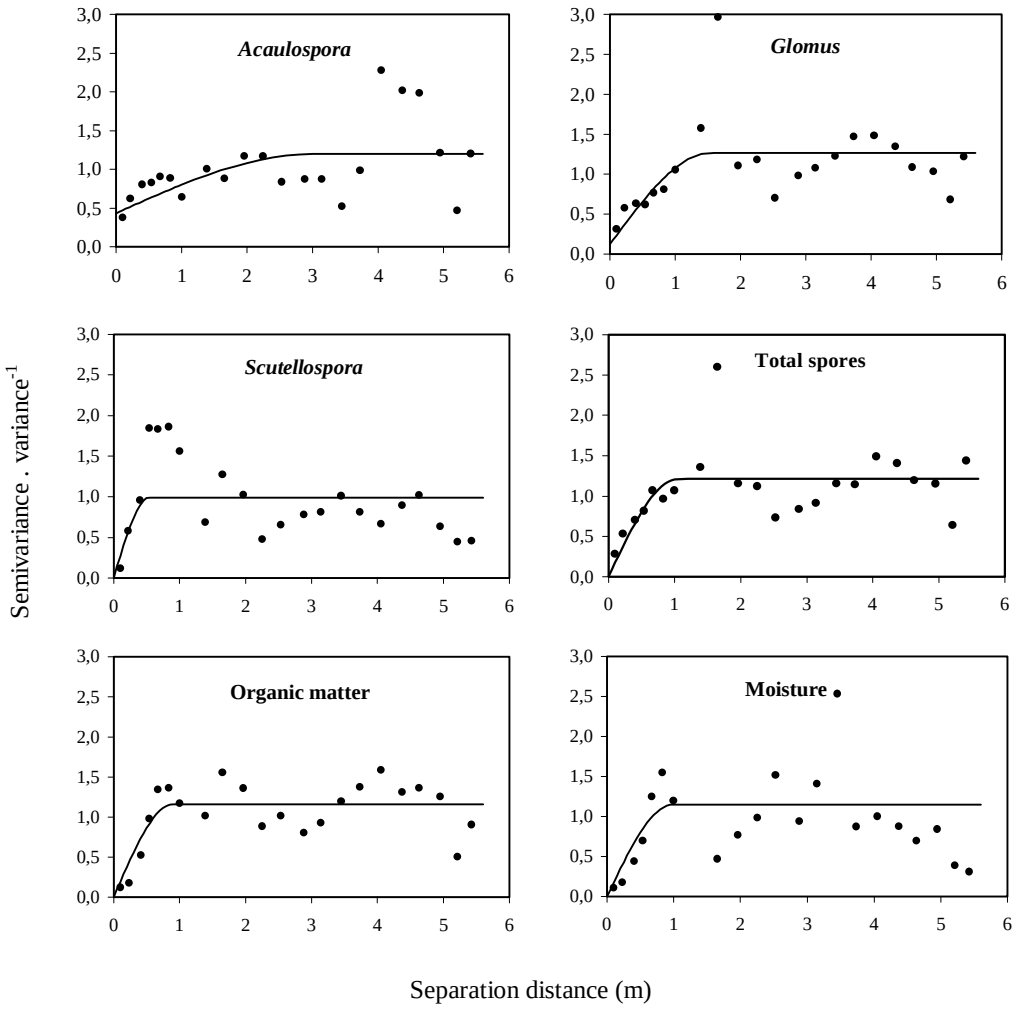

Figure 2. Semivariograms for spore density of *Acaulospora*, *Glomus*, *Scutellospora* and total spores, soil organic matter and soil moisture in maquis vegetation. Standardized semivariance was calculated by dividing semivariance by sample variance. Sample values were ln-transformed prior to semivariance and variance calculation. See Table 3 for parameter descriptions and values.

Based on the coefficient of variation for samples collected in the large grid, there was relatively high variability among patches for spore density and for the soil parameters (Table 2). This suggests that spatial variability among patches was high for both spores and for soil moisture and organic matter. The coefficient of variation for *Glomus* spore density in the salt marsh was nearly identical to that found in the maquis (Table 2), indicating high variability among patches. Soil moisture and organic matter, however, were much more uniform in value (Table 2) in the salt marsh than in the maquis.

Relationships between spore densities, and soil and plant variables were calculated for samples collected in the macrogrid (5 × 5 m). In the maquis, spore densities were positively correlated with organic matter (Table 4). In contrast, there was no significant correlation

between the density of AMF spores and soil moisture. For the salt marsh, the density of AM fungal spores was not significantly correlated with either of the soil parameters studied (Table 4). Correlation between the density of AM fungal spores and plant location was only found in salt marsh (Table 4). The density of *Glomus* spores in salt marsh (which represented all spores) showed a significant positive correlation with the areas of the closest AM plant stands and a highly significant negative correlation with the distance to the closest AM plant stands.

Table 4. Spearman rank-correlations coefficients between spore density of AM fungal genera and soil parameters (organic matter and moisture) and plant parameters (area of closest AM plant, in maquis, or AM plant stand, in salt marsh, and distance to the closest AM plant, in maquis, or AM plant stand, in salt marsh) for the samples collected on the 5 × 5 m 'macrogrid' in salt marsh and maquis (n = 25)

Site / AMF genus	Organic matter	Moisture	Area of closest AM plant or stand	Distance to closest AM plant or stand
Salt marsh				
Glomus	0.069	0.021	0.486*	-0.514**
Maquis				
Acaulospora	0.429*	0.317	0.155	0.186
Glomus	0.435*	0.295	-0.040	0.113
Scutellospora	0.489*	0.311	-0.070	0.279
Total spores	0.480*	0.328	0.005	0.149

* $P < 0.05$; ** $P < 0.01$.

Discussion

In both sites a high degree of variability in AM fungal spore density was found within the sampled areas. In addition, nugget variance was relatively low and spatial dependence rather high (63.8-99.9%) in both sites. As found in most natural ecosystems, the geostatistical analyses indicated that spores were not randomly distributed, but instead spatially distributed in patches, independent of the type of vegetation and AM fungal genus. Soil characteristics for both ecosystems were also found to exhibit spatial heterogeneity, but the variability among patches was much higher in the maquis than in the salt marsh site. The size of spore patches was found to vary with AM fungal genus in the maquis vegetation. Other studies have found highly variable spatial distributions of spores among different AM fungal genera (Eom et al., 2000; Friese and Koske, 1991; Klironomos et al., 1999). These differences in spatial variability may be related to ecological features of AMF. It was recently shown that dispersion of hyphal networks vary among AM fungal taxa (Hart and Reader, 2002), probably

leading to different spatial patterns of sporulation and spore dispersion, and consequently, different patch sizes.

This study suggests that the spatial distribution of spores of AMF differs in the studied plant community types, perhaps reflecting differences in plant composition and plant distribution. The distribution of AM host plant species appears to be an important factor determining the spatial distribution of spores in the salt marsh plant community, as evidenced by the correlation between spore density and distance to *Aster tripolium* plants and also between spore density and projected area of *Aster tripolium* stands. This was likely due to the clumped pattern of AM host plants within non-host plants. On the other hand, in maquis vegetation no relationship was found between AM host plant distribution and spore distribution. This could be due to differences in rooting patterns between the various plant types represented in this community. Although no evaluation was done in our study relating possible differences in rooting patterns of AM host plant, we believe from the above-ground plant distribution that roots from different plant species intermingle (and as a result different mycorrhizal types mix), even at small scales. Therefore, clumps of AM fungal spores are not expected to be present only proximal to AM plants. In salt marsh, the relationship between distribution of AM plants and AM fungal spores strongly suggests that areas of higher spore density are more predictable than in maquis. If spores are a significant form of AM fungal propagation in the salt marsh, then the clumped distribution of plants is likely to produce a mosaic of levels of fungal infectivity within the community, as affected by the location of patches of AM and non-mycorrhizal plants. This suggests that roots of new plants of AM hosts exploiting zones dominated by non-mycorrhizal species would be exposed to low densities of AM fungal inoculum (Carvalho et al., 2001). Spores in this salt marsh seem to be an important propagule since higher density and undamaged appearance of spores was found and previous work in the same site showed high spore viability (Carvalho et al., 2001). In contrast, in maquis a lower density of AM fungal spores and their damaged appearance suggest, at least at the time of sampling, that spores may have little importance for AM fungal propagation. However, this is likely to be true for other seasons, since low viability and relative importance of spores to root infection have been reported for Mediterranean ecosystems (e.g., Requena et al., 1996).

Our results indicated that soil properties might also have an effect on the spatial distribution of AM fungal spores; however, this relationship was site dependent. Organic matter was correlated to the distribution of spores only in the maquis and was independent of AM fungal genera. Although this significant relationship does not necessarily imply a causal

relationship, organic matter has been shown to be positively related to spore production and density in some ecosystems (e.g., Klironomos et al., 1993; St. John et al., 1983). In contrast, organic matter was apparently not an important influence on the spatial distribution of spores in the salt marsh. Since plants and soils influence each other, the influence of either on AM fungal spore distribution cannot always be clearly separated. As plant location was related to spore distribution in salt marsh any possible influence of organic matter in spore spatial distribution could be misleading.

A variety of statistical methods were employed in this study to characterize the distribution of spores and relationships of spore density to edaphic characteristics and plant distribution. Semivariogram analysis was effective in defining the relative size of patches of spores, while coefficient of variation characterized the variability among patches. Correlation analysis aided in identifying physical and biological parameters which may affect spore density. Differences in spore densities and distributions could easily be discerned between the two sites. A similar set of analyses were used by Jackson and Caldwell (1993a,b) and Ryel et al. (1996) to characterize the spatial distribution of nutrients. We suggest that future collection and analysis of data concerning mycorrhizal spore density and spatial distribution follow similar sampling design and statistical analysis to enhance the synthesis and ultimate understanding of factors affecting spore distributions among different plant communities. Although sampling single plots in each plant community in our study was intensive and the isolation and identification of spores was laborious, replicate plots or larger plots within a community could strengthen the analyses. This is most important when the distance necessary for samples to be independent (A_0, Table 3) is similar in size to the plot dimensions. When autocorrelation between sample locations only occurs over distances much smaller than plot dimensions, this is much less an issue, but multiple plots can still address concerns of pseudoreplication (Hurlbert, 1984). In addition, sampling is recommended during different periods of the year when significant within-year changes in distribution of soil attributes occur due to plant and soil processes (e.g., Ryel et al., 1996) and to feedbacks between plants and the microbial community (e.g., Klironomos, 2002; van der Heijden et al., 1998).

Though limited to two ecosystems, and one sampling time, our study suggests that spatial distribution of AMF can vary with plant community type. However, the interpretations should not be conclusively extrapolated to other locations and seasons. The results from two very contrasting plant communities regarding composition and spatial organization of vegetation reported here, provide further insights into the hypothesis that plant community structure can influence the relationship between plants and the distribution of AM fungal spores. Further

research in plant communities with different levels of structure and in different seasons will be important to obtain a better understanding whether and how plant community structure affects the distribution of AM fungal spores.

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CAPÍTULO 4

Arbuscular mycorrhizal fungal propagules in a salt marsh

Este capítulo constitui integralmente o seguinte artigo:

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Arbuscular mycorrhizal fungal propagules in a salt marsh

Luís M. Carvalho · Patrícia M. Correia · M. Amélia Martins-Loução

Centro de Ecologia e Biologia Vegetal, Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal.

Abstract

The tolerance of indigenous arbuscular mycorrhizal fungi (AMF) to stressful soil conditions and the relative contribution of spores of these fungi to plant colonization were examined in a Portuguese salt marsh. *Glomus geosporum* is dominant in this salt marsh. Using tetrazolium as a vital stain, a high proportion of field-collected spores were found to be metabolically active at all sampling dates. Spore germination tests showed that salt marsh spores were not affected by increasing levels of salinity, in contrast to two non-marsh spore isolates, and had significantly higher ability to germinate under increased levels of salinity (20‰) than in the absence of or at low salinity (10‰). Germination of salt marsh spores was not affected by soil water levels above field capacity, in contrast to one of the two non-marsh spore isolates. For the evaluation of infectivity, a bioassay was established with undisturbed soil cores (containing all types of AM fungal propagules) and soil cores containing only spores as AM fungal propagules. Different types of propagules were able to initiate and to expand the root colonization of a native plant species, but spores were slower than mycelium and/or root fragments in colonizing host roots. The AM fungal adaptation shown by this study may explain the maintenance of AMF in salt marshes.

Key words Arbuscular mycorrhizal fungi · Infectivity · Propagules · Salt marsh · Spores

Introduction

Salt marshes are stressful environments regularly flooded with salt water, which leads to high soil salinity and soil anoxia (Armstrong et al. 1985). Although mycorrhizal colonization has been found in salt marsh plants (e.g. Hoefnagels et al. 1993; Carvalho et al. 2001; Hildebrandt et al. 2001), little is known about the survival and infectivity of arbuscular mycorrhizal fungi (AMF) in these harsh ecosystems. One potential mechanism maintaining AMF in salt marshes is fungal tolerance to salt and flooded conditions, which may be species or origin specific. Identification of salt marsh spores has revealed that apparently few AM fungal species occur and that one species, *Glomus geosporum*, is usually dominant in European salt marshes (Carvalho et al. 2001; Hildebrandt et al. 2001; Landwehr et al. 2002). These findings suggest fungal adaptation to salt marsh conditions.

The maintenance of AMF in ecosystems is dependent on the persistence of an inoculum potential in soils (Brundrett 1991). The sources of inoculum of AMF contributing to the infectivity of a soil are spores, infected root fragments and extraradical mycelium. The relative contribution of each type of propagule to plant root colonization is difficult to determine (Smith and Read 1997). There is evidence that this differs among taxa of AMF (Klironomos and Hart 2002) or among habitats, linked generally to differences in their behaviour in response to environmental conditions (Braunberger et al. 1996; Requena et al. 1996; McGee et al. 1997). The tolerance strategies of AMF to adverse salt marsh conditions may include the existence of tolerant propagules, able to maintain an infective inoculum. To our knowledge, no studies have assessed the contribution of the different types of propagules in salt marshes.

There is growing evidence that high spore number can be reached in salt marshes (e.g. Brown and Bledsoe 1996; Hildebrandt et al. 2001; Landwehr et al. 2002). In a salt marsh of the Tagus estuary (Portugal), a high number of spores were metabolically active, regardless of the marsh zones, the levels of salinity or the tidal flooding regimes. In this marsh, spore abundance and distribution seemed to be more related to plant distribution rather than to soil properties (Carvalho et al. 2001, 2003b). These data led us to suspect that spores have a relevant contribution to plant colonization and to the persistence of AMF in salt marshes of the Tagus estuary.

In this present work, we studied the propagules of indigenous AMF from an undisturbed salt marsh by evaluating the metabolic activity of spores at different times and the germination of spores at a range of salinity and water levels, and compared the infectivity of different types of propagules. Specifically, we aimed to evaluate the tolerance of indigenous AMF to salt marsh conditions and to estimate the relative contribution of AMF indigenous spores to plant colonization.

Materials and methods

Study site

Soil samples were collected from the Pancas salt marsh located within the Tagus estuary, Portugal (Carvalho et al. 2001). Some of the plant species present, such as *Aster tripolium* L., *Inula crithmoides* L. and, at a very low level, *Puccinellia maritima* (Huds.) Parl. are

mycorrhizal. Of these, only *A. tripolium* occurs in both the lower and higher marsh zones (Carvalho et al. 2001). Rhizosphere soil samples for each experiment were collected in the lower marsh zone within *A. tripolium* stands.

Field spore vital staining

Soils were collected in July 1997, April 1998, November 1999, and April and November 2000 and stored at 4ºC for 4 months. Spores of AMF were isolated by wet sieving followed by sucrose gradient centrifugation (Daniels and Skipper 1982). From each core sample (7 cm in diameter and 18 cm long), 100 g of soil was sieved and the fraction collected in the last sieve (53 μm) was centrifuged in a 60% (w/v) sucrose solution for 2 min at 3,000 rpm. Spores were collected from the water-sucrose interface, poured through a sieve, rinsed with distilled water and transferred to a Petri dish. Isolated spores were examined under a dissecting microscope at ×45 magnification. The predominant spores from this salt marsh were identified as *Glomus geosporum* (Nicol. and Gerd.) Walker, accounting for 84% of the total spore population, and *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe; two further unidentified *Glomus* species were also present (Carvalho et al. 2001). Forty spores from the two predominant species were collected in proportions representative of those found in the field, placed in iodonitrotetrazolium (INT) solution (1 mg ml^{-1}) and left at room temperature for 48 h (Walley and Germida 1995). Spores were checked for viability and the results expressed as percentage of stained spores. Five samples from each sampling date were collected and processed.

Spore germination tests

The AM fungal spores used in the germination tests originated from collected salt marsh field soils and from cultures of reference non-marsh species. The field soil was collected in April 1998 and stored at 4ºC for 4 months. Two *Glomus* isolates provided by J. Klironomos, originated from the Long-Term Mycorrhizal Research Station at the University of Guelph, Canada and maintained in pot cultures, were used as reference non-marsh spore isolates: namely *Glomus geosporum* and *Glomus mosseae*. These isolates were maintained in our lab as pot cultures with sorghum seedlings in a 1:1 (v/v) mixture of Turface and sand. After 1 year of growth cycling, the pot culture substrates were stored at 4ºC for 4 months.

Spores were tested for germination following the procedure of Weissenhorn et al. (1993). Spores from each isolate type (salt marsh soil-borne, *Glomus geosporum* reference and

Glomus mosseae reference) were isolated by the method described above. Forty healthy spores were collected from each isolate. The sample of salt marsh soil-borne spores represented the two predominant species (*Glomus geosporum* and *Glomus mosseae*) in the proportions found in the field. The spores were placed between two sterilized filter membranes (Gelman, 0.45 μm pore size, 47 mm diameter, grid) held together by a photographic slide frame. This experimental unit with the membranes was buried to half the depth of a glass Petri dish (9 cm diameter) filled with 100 g of sterilized washed river sand (particle size < 1 mm; autoclaved at 110ºC for 1 h on two consecutive days and allowed to stabilize for 2 weeks before use). The sand was watered with the test solution according to each treatment. In the salinity test, the sand was watered to field capacity (25 ml) with NaCl solution at levels of 0‰ (0 mM), 10‰ (171mM), 20‰ (342 mM), 30‰ (513 mM) and 40‰ (684 mM). In the water-level test, distilled water was added to oven-dry (80ºC for 48 h) sand to produce gravimetric water contents of 0 (no water added), 10, 17.5, 25 (soil field capacity level), 37.5 and 50%. The last two levels simulated salt marsh saturated conditions due to flooding. All Petri dishes were sealed with Parafilm to prevent water loss and incubated at 25ºC in the dark. The temperature and time of incubation were based on the results of previous experiments (unpublished data). Each treatment was replicated three times. After 4 weeks, the experimental units were removed from the sand, rinsed with tap water and stained with glycerol-trypan blue solution (0.05%) for 1 h. The two membranes were carefully separated and examined under a dissecting microscope at ×45 magnification. The percentage of germinated spores was assessed.

Assessment of propagule infectivity

Undisturbed soil cores were collected in November 2000 from 12 randomly selected points. At each sampling point, four intact soil cores were taken immediately adjacent to each other with PVC tubes, 9 cm in diameter and 15 cm long. Each soil core remained in the PVC tube. The amount of soil in each core was 945 g (± 38 SE) fresh weight or 481 g (± 19 SE) dry weight.

For the propagule infectivity tests, one core from each one of the 12 sets of four cores remained intact, thus containing all types of AM fungal propagules (‘all-propagule’ treatment). From each set, two cores were used for the ‘spore treatment’. The soil from one core was removed from the PVC tube and AM fungal spores were extracted from the entire soil volume and stored for 24 h in distilled water at 4ºC. The soil from the other core was

removed from the PVC tube, autoclaved at 110ºC for 1 h on two consecutive days and allowed to stabilize for 2 weeks before use. The stored spore solution were mixed with the autoclaved soil and placed into a new PVC core. Thus, each of the two treatments had twelve cores. Of the remaining cores, the soil of six cores was removed, autoclaved and repacked into new PVC tubes (control cores). The other five cores were analyzed for spore density and extraradical hyphal length of AMF. In both propagule treatments (all propagule and spores only), three seedlings of *A. tripolium* germinated on Petri dishes in the dark at 25ºC for 7-10 days were transplanted into each core of soil. The cores with the seedlings were placed randomly in a growth chamber at temperatures between 15 and 24ºC and a photoperiod of 16 h at a photosynthetic photon flux density of 250 μmol m^{-2} s^{-1}. Plants were watered to field capacity with a 50% solution of artificial seawater (adapted from Epstein 1972).

Six replicate cores were harvested from each treatment at 3 or 6 weeks after transplanting. Three of the control cores were also harvested on both dates. At each harvest, the roots were extracted, cleaned and the total length determined by digital image analysis with WinRhizo software (Regent Instruments Image Analysis Systems, Canada). The roots were cleared and stained by a modified Phillips and Hayman (1970) procedure, in which roots were cleared for 50 min in a 10% KOH solution at 90ºC, rinsed, placed in 10% HCl solution for 10 min and then stained with glycerol-trypan blue solution (0.05%) at 90ºC for 20 min. Infection units originating from entry points were counted at ×200 magnification (Franson and Bethlenfalvay 1989). The results were expressed as infection units per root length. Root colonization by AM fungi was estimated by the gridline intersection method at ×45-100 magnification (Giovannetti and Mosse 1980) and expressed as percentage of root length colonized and colonized root length.

In each of the five field soil cores from different sets, spores were isolated from 30 g of soil and expressed as spore number per soil dry weight. Extraradical hyphae were extracted from two soil samples (5 g fresh weight each) by a modified Miller et al. (1995) procedure. Individual soil samples were suspended in 495 ml of water and homogenized in a blender for 1 min. The suspension was decanted and then stirred with an electronic stir bar. One 20-ml aliquot was removed from halfway between the beaker edge and the vortex and diluted in 80 ml of water. This mixture was stirred again and one 10-ml aliquot was transferred to a filter holder with a nitrocellulose membrane filter (47 mm diameter, 1.2 μm pore size). Trypan blue stain (0.05%) was added and the stained suspension was drawn through the filter using vacuum suction after 5 min. The filter was cut in half and placed on microscope slides. After

drying, the filters were covered with low viscosity immersion oil. Extraradical mycelium was estimated by the gridline intersect method recording 140 fields of view using a 10×10 squared grid eyepiece reticule and viewed at ×200 magnification. Only aseptate hyphae with a characteristic 'knobby' appearance and dichotomous branching were considered as AM fungal hyphae. Total hyphal length was calculated based on Tennant (1975) and expressed as meters per g dry soil on the basis of soil moisture content measurements.

Statistical analysis

All data from the three experiments were analyzed by ANOVA and significant results (P<0.05) were analyzed by Duncan's test. Field spore vital staining data were analyzed by one-way ANOVA to test for differences between sampling dates. Spore germination data were analyzed by two-way ANOVA to test for differences between spore isolates and salinity or water level. Prior to analyses, germination data were arcsin square root-transformed (Zar 1984). Infectivity assessment data were analyzed by two-way ANOVA to test for differences between propagule type and harvest date variables. Prior to analyses, percentage of root length colonized data were arcsin square root-transformed and infection units data were log-transformed (Zar 1984). Statistica software (StatSoft, Tulsa, USA) was used for all statistical analysis.

Results

Field spore vital staining

The percentage of stained field spores was generally high, ranging from a mean of 61 to 80% (Fig. 1). A significant sampling date variation was found ($F_{4,18}$=7.5, P<0.01): the percentage of stained spores was higher in spring and summer than in autumn.

Spore germination tests

In the soil salinity germination tests (Fig. 2), there was a significant spore isolate × NaCl concentration interaction ($F_{8,30}$=6.9, P<0.001) along with significant main effects of the origin of the isolates ($F_{2,30}$=89.3, P<0.001) and NaCl concentration ($F_{4,30}$=15.8, P<0.001). This supports the hypothesis that the isolates exhibit different responses to NaCl concentrations.

Germination of *Glomus geosporum* and *Glomus mosseae* reference spores decreased with increasing NaCl concentrations and spores of these isolates failed to germinate at concentrations higher than 10‰ (*Glomus mosseae*) or 30‰ (*Glomus geosporum*). Spores originating in the salt marsh had significantly higher germination at 20‰ NaCl than at lower concentrations, and germination decreased slightly with higher concentrations. However, at the highest concentration tested, germination of spores was not significantly different from that at the lowest concentrations (0 and 10‰). Germination of salt marsh spores was significantly higher than reference isolates at NaCl concentrations higher than 0‰.

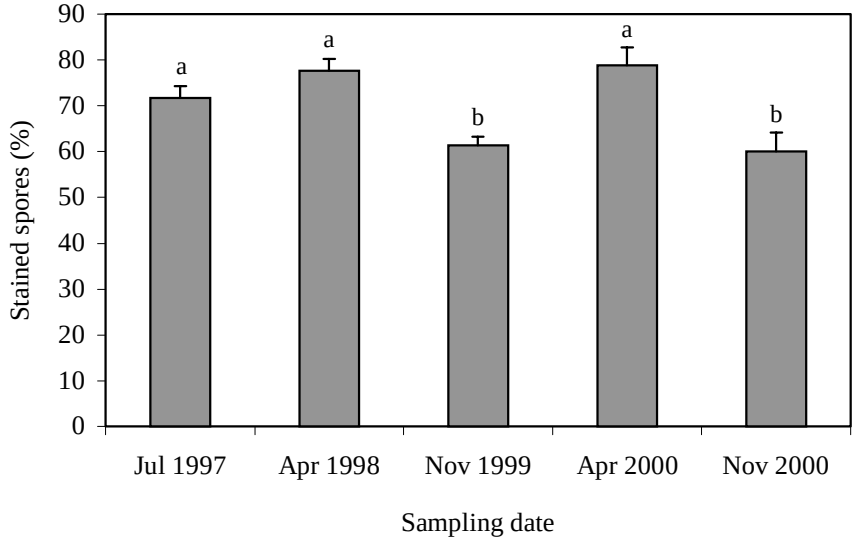

Fig. 1 Vital staining of field spores collected from Pancas salt marsh. Values (means ± SE of 5 replicates) followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

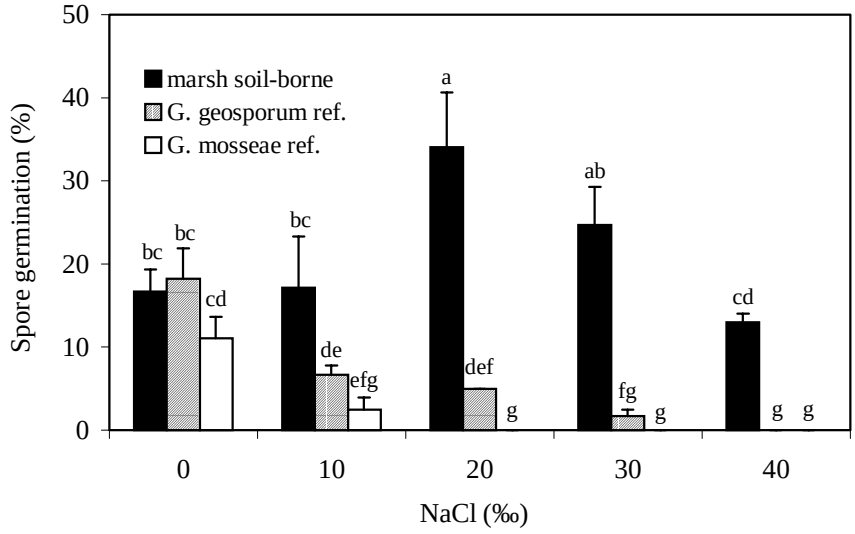

Fig. 2 Germination of spores collected from Pancas salt marsh and reference non-marsh spores of *Glomus geosporum* and *Glomus mosseae* under different soil salinity levels (NaCl concentrations). At each NaCl concentration, values (means ± SE of 3 replicates) followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

In the soil water level germination tests (Fig. 3), besides the significant main effects of spore origin ($F_{2,34}$=3.6, P<0.05) and water level ($F_{5,34}$=34.5, P<0.001), there was a significant spore isolate × water level interaction ($F_{10,34}$=3.5, P<0.01), indicating differences in the responses of the different isolates to water levels. All spores failed to germinate without water in the incubation soil. Soil water content below field capacity (<25%) significantly reduced germination of salt marsh spores and *Glomus geosporum* reference spores, but not *Glomus mosseae* spores. Germination of salt marsh spores was not significantly affected by increased soil water contents (above field capacity) as compared to non-marsh spores, in particular to the *Glomus geosporum* reference isolate. At the highest soil water level tested, salt marsh spores had higher germination than non-marsh spores, although this was only significant for the *Glomus mosseae* reference isolate.

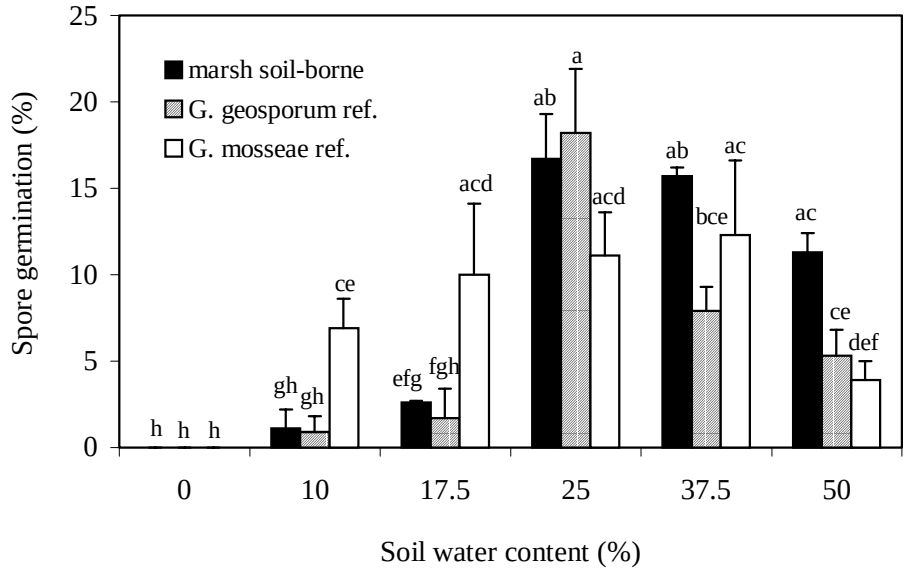

Fig. 3 Germination of spores collected from Pancas salt marsh and reference non-marsh spores of *Glomus geosporum* and *Glomus mosseae* under different gravimetric soil water contents (g H_2O per 100 g dry-soil). At each water level, values (means ± SE of 3 replicates) followed by the same letter are not significantly different at P<0.05 (Duncan's test)

Assessment of propagule infectivity

An average of 4.7 m g^{-1} soil dry weight (± 0.8 SE) extraradical AM fungal mycelium and 3.8 spores g^{-1} soil dry weight (±0.6 SE) were found in salt marsh soil.

The significant type of propagules (all propagules vs. spores only) × harvest period interactions for percentage of root length colonized, colonized root length and number of infection units, showed that the differences in colonization between propagules treatments were not consistent in the two harvest periods (Table 1). Colonization started earlier in the all-

propagule treatment than in the spore treatment. Roots were colonized in all cores of the all-propagule treatment at 3 weeks, while only half of the cores displayed root colonization in the spore treatment. After 3 weeks of plant growth, root length colonization and infection units were significantly higher in the all-propagule treatment than in the spore treatment, where those parameters were extremely low. In the spore treatment, infection units and colonization of root system increased significantly with time, in contrast to the all-propagule treatment. In the latter, a significant decrease in the number of infection units occurred with time, probably due to the coalescence of many entry points. After 6 weeks of plant growth, colonized plant frequency and root length colonization were similar in the two treatments. An increase in the number of infection units was detected over time in the spore treatment. However, the infection units recorded at 6 weeks in the spore treatment appeared to be lower, but not significantly different, than those detected in the all-propagule treatment at 3 weeks. The significant difference in the percentage of root length colonization between propagule treatments at each harvest was reflected in the colonized root length, indicating that the dissimilarity was not due to difference in the total root length of host plants. Shoot dry weight of *A. tripolium* plants was not significantly influenced by propagule type treatment (data not shown). At each harvest date, neither root colonization nor infection units were observed in plants of the control cores.

Table 1 Frequency of soil cores with colonized roots, percentage of root length colonized, colonized root length and number of infection units per meter of root in the intact soil cores (all types of propagules) and in the spore soil cores, after 3 and 6 weeks of *A. tripolium* growth. *F*-values with significance levels are given for a two-way ANOVA. Degrees of freedom was 1 for each source of variation (propagules, harvest period and their interaction). Values (means ± SE, n = 6 replicates) within each column followed by the same letter are not significantly different at $P<0.05$ (Duncan's test)

Type of propagules and harvest time	Colonized cores (%)	Root length colonized (%)	Colonized root length (cm)	Infection units
3 weeks				
All	100	24±7 a	15±4 b	283±93 a
Spores	50	1±1 b	1±0 c	3±1 c
6 weeks				
All	100	22±12 a	41±28 ab	82±44 b
Spores	100	21±7 a	50±17 a	62±26 b
Source of variation				
Propagules		5.5*	3.3	17.3**
Harvest period		4.1	26.0***	1.3
Propagules×harvest		7.3*	15.0**	21.6***

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Discussion

In this study, we found evidence for potential adaptation of indigenous AMF to salt marsh conditions and for the ability of different propagules of these fungi to infect new plants and spread the infection through the roots.

In the Pancas salt marsh, the results of the vital staining tests showed a high proportion of metabolically active spores at different time periods and also when compared to other types of ecosystems (e.g. McGee et al. 1997; An et al. 1998). The high abundance (Carvalho et al. 2001) and metabolic activity of spores indicates a substantial spore pool throughout the year in salt marsh soils, with a high proportion of spores likely to germinate. However, the proportion of spores that germinated in the tests was considerably lower than the proportion of field spores, from the same collection date, identified as viable by the vital staining test. If the experimental conditions of the germination tests were conducive to maximal germination, then possible explanations for the discrepancy include the staining of dormant spores and/or the cytoplasm of immature spores that do not germinate. Although tetrazolium staining can be a useful and fast procedure to assess spore metabolic activity, we suggest precaution in the interpretation of results when it is used to estimate the potential of spores to germinate, in agreement with other reports (McGee et al. 1997).

Spore germination tests showed that spores from the salt marsh were more tolerant to salinity than spores of non-marsh reference isolates and required NaCl levels of 20 or 30‰ for maximal germination. This corresponds to similar or higher salinity levels found in the Pancas salt marsh (Carvalho et al. 2001). Koske et al. (1996) also observed salt tolerance of sand dune spores of *Gigaspora gigantea*, while other studies showed a strong inhibition by sodium and chloride salts of spore germination in *Gigaspora margarita* (Hirrel 1981) and *Glomus mosseae* (Estaun 1989). We also observed a strong reduction in germination by these salts in the reference isolate of *Glomus mosseae*.

Germination of salt marsh spores was not affected by water level at field soil capacity or above but decreased below it, probably due to water stress. Similar observations were reported by Daniels and Trappe (1980) for *Glomus epigaeum* and by Koske (1981) for *Gigaspora gigantea*. However, reduced spore germination in soils with water levels above field capacity, as we observed for the reference isolate of *Glomus geosporum*, has been observed for some species of *Glomus* (Sylvia and Schenck 1983). The reduced germination at high water levels may be related to the low tolerance of some fungal species of hypoxic conditions.

Since AMF are obligate symbionts, spore germination and subsequent early hyphal growth of hyphae are the only stages of AM fungal cycle in which the fungi can be studied in the absence of plants. Therefore, spore germination is a good predictor to test the influence of certain factors on AMF. The ability of salt marsh spores to germinate under high salinity and above soil field capacity suggests that the AMF present in the salt marsh soil are probably AM fungal ecotypes adapted to the stressful conditions present in this ecosystem. Similarly, Landwehr et al. (2002) suggested the occurrence of *Glomus geosporum* ecotypes in Central European salt marshes.

The results of the infectivity tests showed that different types of propagules of salt marsh AMF are able to initiate new colonization and to spread the infection through the root system of a native plant. Mycelium and/or root fragments were relatively more important for the initiation of plant colonization than spores, at least in the experimental conditions used. Since spores required from 3-6 weeks to initiate colonization in the spore treatment, we conclude that the infection units and colonization of roots harvested at 3 weeks in the all-propagule treatment were due to non-spore propagules. We do not know the relative proportion of infection units derived from extraradical mycelium or root fragments. But it is reasonable to suggest that mycelium was an important propagule for the initiation of colonization. In spite of the stressful environment, salt marsh soil contained a considerable hyphal network. Previous reports have indicated that hyphal networks are a very important source for the rapid initiation of colonization (McGee et al. 1997; Smith and Read 1997). Low abundance and viability of spores may sometimes explain the failure of spores to rapidly initiate colonization (McGee 1989; Requena et al. 1996), but this was not evident in the present study. The inherent time required for the transition from dormancy to germination, which is a highly variable character in the Glomales (Tommerup 1983), may have accounted for the slower initiation of infection by spores than by the hyphal network.

Salt marshes are subjected to large temporal and spatial variation of soil properties (Armstrong et al. 1985). High levels of salinity and flooding in soils were observed to significantly reduce the extraradical mycelium length of AMF from the Pancas salt marsh (Carvalho et al. 2003a). In the present investigation, colonization levels due to spores were similar to those found for field plants (Carvalho et al. 2001). This result and the large proportion of active spores indicate that the pool of spores is likely sufficient to maintain the usual levels of AM colonization in salt marsh plants. Although our data do not indicate that spores make a large contribution to the initiation of colonization, we can not completely exclude that spores play a significant role in salt marshes. Further studies testing the effects of

season, salinity and flooding on the infectivity of each type of propagule are necessary to determine whether spores, as resistant structures, function as survival units of AMF in salt marshes.

The results of this study showed that fungal adaptation is one potential mechanism to explain the maintenance of AMF in the stressful salt marsh soils. The specific adaptation and tolerance to salinity and water levels of the AMF from the studied salt marsh may provide an explanation for the low fungal diversity and wide occurrence of AMF in marsh zones, regardless of the tidal flooding regime (Carvalho et al. 2001). These salt marsh AMF may have the potential to confer salt and flooding tolerance to plants and, therefore, may influence plant distribution in salt marshes.

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CAPÍTULO 5

Effects of salinity and flooding on the infectivity of salt marsh arbuscular mycorrhizal fungi in *Aster tripolium* L.

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Effects of salinity and flooding on the infectivity of salt marsh arbuscular mycorrhizal fungi in *Aster tripolium* L.

Luís M. Carvalho · Patrícia M. Correia · Isabel Caçador · M. Amélia Martins-Loução

Luís M. Carvalho · M. A. Martins-Loução
Centro de Ecologia e Biologia Vegetal, Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal.
Isabel Caçador
Instituto de Oceanografia, Faculdade de Ciências de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

Abstract

Salt marshes are characterized by the occurrence of combined salinity and flooding stresses. The individual and combined effects of salinity and flooding on the establishment and activity of arbuscular mycorrhizal (AM) colonization in the salt marsh halophyte *Aster tripolium* L. by indigenous salt marsh AM fungi were evaluated. *A. tripolium* plants were cultivated in a mixture of sand and salt marsh soil under different salinity concentrations (5%, 50% or 100% artificial seawater) and water regimes (non-flooding, tidal flooding and continuous flooding). Plants were harvested after 3 and 8 weeks and their growth was negatively influenced by increased salinity and water level. Increased salinity level affected the establishment of AM colonization, AM fungal growth and activity (measured as succinate dehydrogenase activity) within roots, and extraradical mycelium growth. The influence of flooding on the establishment of colonization and on intra- and extraradical AM fungal growth was dependent on the water regime. Continuous flooding reduced colonization and AM fungal growth, whereas tidal flooding did not affect these parameters unless combined with intermediate salinity level (50% seawater) at the end of the experiment. The water regime did not influence AM active colonization. The ratio of root to soil AM fungal growth increased as the water level increased. The results of this study demonstrate that the establishment and activity of AM colonization in *A. tripolium* is more influenced by salinity than by flooding, and suggests that the functionality of salt marsh AM fungi is not affected by flooding.

Key words Arbuscular mycorrhiza · *Aster tripolium* · Flooding · Salinity · Succinate dehydrogenase activity

Introduction

Salt marshes are among the most productive ecosystems in the world and have an important role as sinks for heavy metals and nutrients (Adam 1990). In these habitats the regular inundation with seawater leads to high soil salinity and flooding with subsequent soil anoxia (Armstrong et al. 1985; Pennings and Callaway 1992). An accelerated sea level rise as a result of global climate changes will expose salt marshes to the increasing frequency and duration of tidal inundation (Adam 2002). This will induce changes in the soil properties associated with

tidal inundation, such as salinity, redox potential, nutrient levels and structure. Plants and soil organisms will have to cope with this additional intensity of stress in the soil environment. An understanding of how plants may respond to a rising sea level is incomplete without considering plant-associated soil organisms. Mutualistic associations with soil microorganisms, such as arbuscular mycorrhizas, may improve plant tolerance to those stressful conditions because of the recognized role that mycorrhizas have in plant performance. However, knowledge on the extent to which salinity and flooding affect the growth and infectivity of indigenous arbuscular mycorrhizal fungi (AMF) is scarce.

It has been shown that salinity can affect the growth of AMF and their root colonization in various ecosystems (Juniper and Abbott 1993; McMillen et al. 1998; Al-Karaki 2000; Cantrell and Linderman 2001). However, others works did not find a reduction in root colonization with salinity (Copeman et al. 1996; Ruiz-Lozano et al. 1996). Ruiz-Lozano and Azcón (2000) reported that salt effects on AM might differ with either the species or the origin of the fungus. Flooding has also been shown to decrease or partially inhibit AM fungal root colonization (Muthukumar et al. 1997; Miller 1999; Miller and Sharitz 2000).

In salt marshes, organisms are subjected to the combination of salinity and flooding stresses. The assessment of multiple environmental effects on AMF and plants is therefore important to obtain a more meaningful and realistic view of the role of mycorrhizas in salt marshes. The combined effects of these two stresses on AM fungal growth and infectivity are very poorly understood. Furthermore, it is important to take into account that in salt marshes, plants are in general inundated periodically by tidal rather than by permanent flooding. Therefore, tidal flooding may promote different AM responses relatively to continuous flooding. To our knowledge, no studies have previously addressed the question of the influence of tidal flooding on arbuscular mycorrhizas.

The halophyte *Aster tripolium* L. is a common perennial herb and one of the pioneer species in many European salt marshes. In a salt marsh of the Tagus estuary (Portugal) with very few mycorrhizal plant species, *A. tripolium* was the only one present in both marsh zones (less and more flooded). However, the extent of AM colonization in its roots did not vary among marsh zones (Carvalho et al. 2001). Germination of spores of that salt marsh was neither affected by increasing levels of soil salinity nor by increasing levels of soil moisture (Carvalho et al. 2003). These results indicate that AMF present in the salt marsh soil may have the potential to tolerate salinity and flooded conditions.

The aim of this work was to assess the individual and combined effects of salinity and flooding on the initiation and spread of AM colonization in *A. tripolium* by indigenous salt

marsh AMF. We also aimed to evaluate intraradical AM fungal activity to discriminate between possible different effects of salinity and flooding on active *vs*. inactive parts of the fungi. To accomplish these goals, we measured total (non-vital staining technique) and active root colonization [vital staining of succinate dehydrogenase (SDH) activity], and AM fungal extraradical mycelium (ERM) under different levels of salinity (artificial seawater concentrations) and different water regimes (non-flooding, tidal flooding and continuous flooding) in a factorial design.

Materials and methods

Soil and mycorrhizal inoculum

The soil used as mycorrhizal inoculum was collected from Pancas salt marsh located within the Tagus estuary in Portugal. This salt marsh is tidally inundated by salt water with a semi-diurnal regime. Soil was collected within *Aster tripolium* stands and had a pH (H_20) of 6.0, 11.9% organic matter, 97% clay, 2 g total N kg^{-1} and 900 mg extractable P kg^{-1}. This soil was used as mycorrhizal inoculum and AM fungal species composition consisted only of *Glomus* species, with *G. geosporum* (Nicol. and Gerd.) Walker as the most abundant species (Carvalho et al. 2001). The soil was stored at 4ºC until used. The washed river sand (<1 mm) used in this experiment was autoclaved at 110ºC during 1 h on 2 consecutive days.

Plant material

Sterilized seeds of *A. tripolium* collected in autumn from Pancas salt marsh were germinated on Petri dishes lined with moistened filter paper and placed in the dark at 25ºC for 7-10 days. Uniform seedlings were then transferred to plastic containers (40×28×7.5 cm) containing autoclaved sand and they grew in a controlled environmental growth room, with day and night temperatures of 25ºC and 18ºC, respectively, a photoperiod of 14 h at a quantum flux density of 400 μmol m^{-2} s^{-1} and around 50% relative humidity. The plants were watered daily to field capacity with deionized water and received 1/4-strength Hoagland modified solution (Hoagland and Arnon 1939) with a reduced P concentration (0.1 mM) once a week.

Experimental design

To test the effects of salinity and flooding on the initiation and spread of colonization on *A. tripolium* by salt marsh AMF, a 3×3 factorial experimental design with three levels of salinity (low, intermediate and high) and three water regimes (non-flooding, tidal flooding or continuous flooding) was established. After 4 weeks of establishment, 108 *A. tripolium* plants were individually transplanted to 250-ml plastic pots (8 cm diameter) filled with a 4:1 (v/v) mixture of autoclaved sand and non-sterile salt marsh soil. Each potted plant was placed in a larger plastic container. Twelve replicate pots were assigned to each of nine treatments and arranged in a completely randomised design. The plants grew in the same controlled environmental growth room described above and the pots were randomly rotated at weekly intervals to avoid site effects within the growth room.

Salinity levels were achieved by adding artificial seawater in concentrations of 5 (low salinity), 50 (intermediate salinity) and 100% (high salinity). We used artificial seawater solution instead of NaCl solution, because it is a more representative of natural conditions in salt marshes. The low salinity level chosen was 5% seawater instead of 0%, because salt is always present in salt marsh environments. Full-strength artificial seawater solution (100%), adapted from Epstein (1972) to salt marsh systems, contained: 410.52 mM NaCl, 9.93 mM KCl, 10.23 mM $CaCl_2$, 53.58 mM $MgCl_2$, 28.25 mM Na_2SO_4, 2.34 mM $NaHCO_3$, 0.83 mM NaBr, 0.07 mM $SrCl_2$ and 0.44 mM H_3BO_3. A 50% and 5% seawater concentration were applied by appropriate dilutions of full-strength artificial seawater. To avoid osmotic shock in the 100% seawater treatments, it was applied as 50% seawater concentration at the beginning and it was only increased to 100% after 2 days.

In the water regime treatments, tidal flooding was chosen because it simulates what happens at Pancas salt marsh and continuous flooding represents the extreme conditions. The non-flooded pots were watered daily to field capacity with deionized water or seawater solution as necessary. In the tidally flooded treatments a system device was set up to simulate a tidal regime of 1 h of flooding at 20-h intervals. A pump connected to a programmed timer was used to transfer artificial seawater solution of each one of the three different seawater concentration tanks to the respective pots, simulating tidal action. A 2-cm inundation level above the pots was maintained during flooding (a small hole in the containers released the excess of water). Flooding in the continuously flooded treatments was achieved by filling the containers with the respective seawater solution until reaching a level of 2 cm above the soil surface, which was maintained during the experiment. The solutions in the continuously

flooded treatments were aerated, by bubbling air during the same 1-h period at 20-h intervals in the tidally flooded treatments, since even in very flooded salt marsh zones there is some soil aeration due to tidal action. In the tidally and continuously flooded treatments seawater solutions were renewed every week. Every 2 weeks 1/4-strength Hoagland solution with a reduced P concentration (0.1 mM) was added to all pots.

A supplementary experiment was conducted to assess the soil redox potential in the three different water regimes. This was not measured in the major experiment, since the introduction of the electrode in the soil would have disturbed and damaged both ERM and roots. For each water regime treatment, six pre-established *A. tripolium* plants grew in the same previously described pots and substrate, with a low seawater concentration (5%). The redox potential measurements were made after 1 and 3 weeks of plant growth and coincided with the gap between two tidal inundations which occurred in the tidal flooding treatment. A platinum electrode was inserted into the soil at a depth of 5 cm and the redox potential was recorded after a stabilization period of 2 min.

Harvest and analysis

Six plants from each treatment were randomly harvested 3 and 8 weeks after water regime and salinity treatments were applied. Total root length was determined through digital image analysis with WinRhizo software (Regent Instruments Image Analysis Systems, Canada). Shoot dry weight was determined after drying at 60ºC for 5 days. At the 3-week harvest, fresh roots were stored in 50% ethanol for subsequent assessment of total colonization. At the 8-week harvest, the fresh root system was divided in two halves: one was also stored in 50% ethanol and the other one was immediately processed for active colonization assessment. Two soil cores were also taken from each pot at the end of the experiment for the quantification of the ERM length. One week before the last harvest, soil interstitial water was taken carefully from each pot, through a system consisting of a long and fine tube connected to a syringe, and salinity was measured with a hand-held refractometer.

Total root colonization was assessed by a modified Phillips and Hayman (1970) procedure (Carvalho et al. 2001), quantified by the gridline intersection method at ×45-190 magnification (Giovannetti and Mosse 1980) and expressed as percentage of total root length colonized and as total colonized root length (m). The staining method with trypan blue does not discriminate active from inactive parts of the fungi. Therefore, AM colonization was also estimated by SDH activity using the nitro blue tetrazolium staining method (Kough et al.

1987), quantified by the gridline intersection method at ×45-190 magnification and expressed as percentage of active root length colonized and as percentage of root mycorrhizal fungi that are metabolically active.

The two soil cores taken from each pot were mixed and 5 g of soil was dried (60ºC, 5 days) for dry weight determination and another 5 g of soil was used to extract ERM by soil homogenisation and dilution, and hyphal staining (Miller et al. 1995). ERM was estimated by the gridline intersect method by examining 140 fields of view at ×200 magnification. Only aseptate hyphae with a characteristic "knobby" appearance and dichotomous branching were considered as AM fungal hyphae. ERM length was calculated according to Tennant (1975) and converted to meters per g dry soil. To determine whether salinity and water regime affect AM fungal growth in roots *vs*. soil, the ratio of intraradical mycelium (IRM) length (colonized root length per gram root fresh weight) to ERM (ERM length in soil per gram soil fresh weight) at the 8-week harvest was calculated.

Statistical analysis

Data of soil redox potential from the supplementary experiment for both measurement periods were pooled because they were not significantly different; then they were analysed with a one-way analysis of variance (ANOVA) to test for differences between water regimes. Data of soil interstitial water salinity, plant and fungal variables from each harvest were analysed separately with a two-way ANOVA to test for effects of salinity level and water regime. Significant results ($P<0.05$) were analysed by Duncan's test. Prior to statistical analysis, data were transformed (arcsin square root-transformed for percentage variables and log-transformed or square root-transformed for other variables) as necessary to satisfy the assumptions of the ANOVA (Zar 1984). Statistica software (StatSoft, Tulsa) was used for all statistical analysis.

Results

Different water regimes significantly affected the redox potential ($F_{2,33}=273.9$, $P<0.001$) measured in the supplementary experiment, indicating more aerated soil conditions in the non-flooding treatment (376±9 mV) and more reduced conditions in the flooding treatments

(145±11 and 93±7 mV for tidal flooding and continuous flooding, respectively) (mean±SE; n=12).

Measures of soil interstitial water salinity differed significantly among seawater (salinity) levels ($F_{2,91}$=486.2, P<0.001), but they did not significantly vary among water regimes ($F_{2,91}$=1.4, P=0.252) and the interaction between seawater level and water regime was not also significant ($F_{4,91}$=0.7, P=0.578). Salinity was 5.2±0.2 ‰, 16.4±0.4 ‰ and 30.5±0.9 ‰ at 5%, 50% and 100% seawater treatments, respectively (mean±SE; n=36).

Table 1 Shoot dry weight *of Aster tripolium* plants after 3 and 8 weeks of growth under different salinity levels (5%, 50% or 100% seawater) and water regimes [non-flooding (*NF*), tidal flooding (*TF*) or continuous flooding (*CF*)]. *F*-values with *asterisk* indicating significance level are shown for a two-way ANOVA. Values (means±SE of six replicates) for each harvest date and within each column followed by *the same letter* are not significantly different at P<0.05 (Duncan's test)

Salinity	Water regime	Shoot dry weight (mg plant^{-1})	
		3 Weeks	8 Weeks
5%	NF	90±7 a	369±15 a
	TF	57±6 bc	250±13 c
	CF	61±6 b	197±12 d
50%	NF	59±6 b	311±21 b
	TF	66±13 b	135±17 e
	CF	40±2 d	110±10 e
100%	NF	27±2 e	92±24 e
	TF	42±4 cd	88±9 e
	CF	28±3 e	93±11 e
Source of variation	*df*	F-values	
Salinity	2	35.1***	103.6***
Water regime	2	5.0*	54.5***
Salinity×water regime	4	4.2*	14.4***

* P<0.05, ** P<0.01, *** P<0.001

Overall, both increased salinity and water level decreased significantly the shoot dry weight of *A. tripolium* at each harvest date (Table 1). However, in the highest salinity level, water regime had generally no effects, resulting in significant salinity×water regime interaction. At the final harvest (week 8), non-flooded plants exposed to an intermediate or high salinity level showed a reduction in shoot dry weight of 16% and 75%, respectively, relative to non-flooded plants of the lowest salinity treatment. At the end of the experiment, in the intermediate salinity treatments the combination with flooding (tidal or continuous) produced greater reductions in shoot dry weight than each factor alone.

Table 2 Summary of two-way ANOVA of the effect of salinity (seawater level) and water regime on AM fungal parameters after 3 and 8 weeks of plant growth. *F*-values with *asterisk* indicating significance level are shown. *ERM* Extraradical mycelium length, *IRM* intraradical mycelium length

Response variable	Source of variation		
	Salinity (*df*=2)	Water regime (*df*=2)	Salinity×water regime (*df*=4)
3 Weeks			
% Total colonization	110.7***	7.6**	1.4
Colonized root length	196.5***	9.0***	4.5**
8 Weeks			
% Total colonization	201.3***	12.7***	4.5**
Colonized root length	276.1***	13.3***	6.3***
% Active colonization	111.1***	0.9	0.8
% Active mycorrhizal fungi	2.0	3.4*	0.8
ERM length	80.8***	45.0***	0.5
IRM/ERM ratio	9.0***	20.0***	0.2

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

There was a significant effect of salinity and water regime on both percentage of total colonization by AMF and total colonized root length at each harvest date (Table 2). In addition there were also significant salinity×water regime interactions. Indeed, water regime differences were not observed at the highest salinity level (Fig. 1a-d). When the salinity was increased to 50%, there was a decrease in percentage of total colonization by AMF, which was more pronounced at 3 than at 8 weeks (Fig. 1a, b). However, when the salinity levels were further increased to 100%, a drastic decrease (nearly inhibition) in the percentage of total colonization occurred, independently of the water regime. Continuously flooded plants had the lowest percentage of total colonization and colonized root length (Fig. 1a-d). Continuous flooding at 50% salinity affected the beginning rather than the spread of colonization (percentage colonization raised 3.5-fold from week 3 to week 8). Tidal flooded plants only displayed a lower percentage of colonization than non-flooded plants when this treatment combined with intermediate salinity at week 8 (Fig. 1a, b). Colonized root length showed similar trends as percentage of total colonization (Fig. 1c, d).

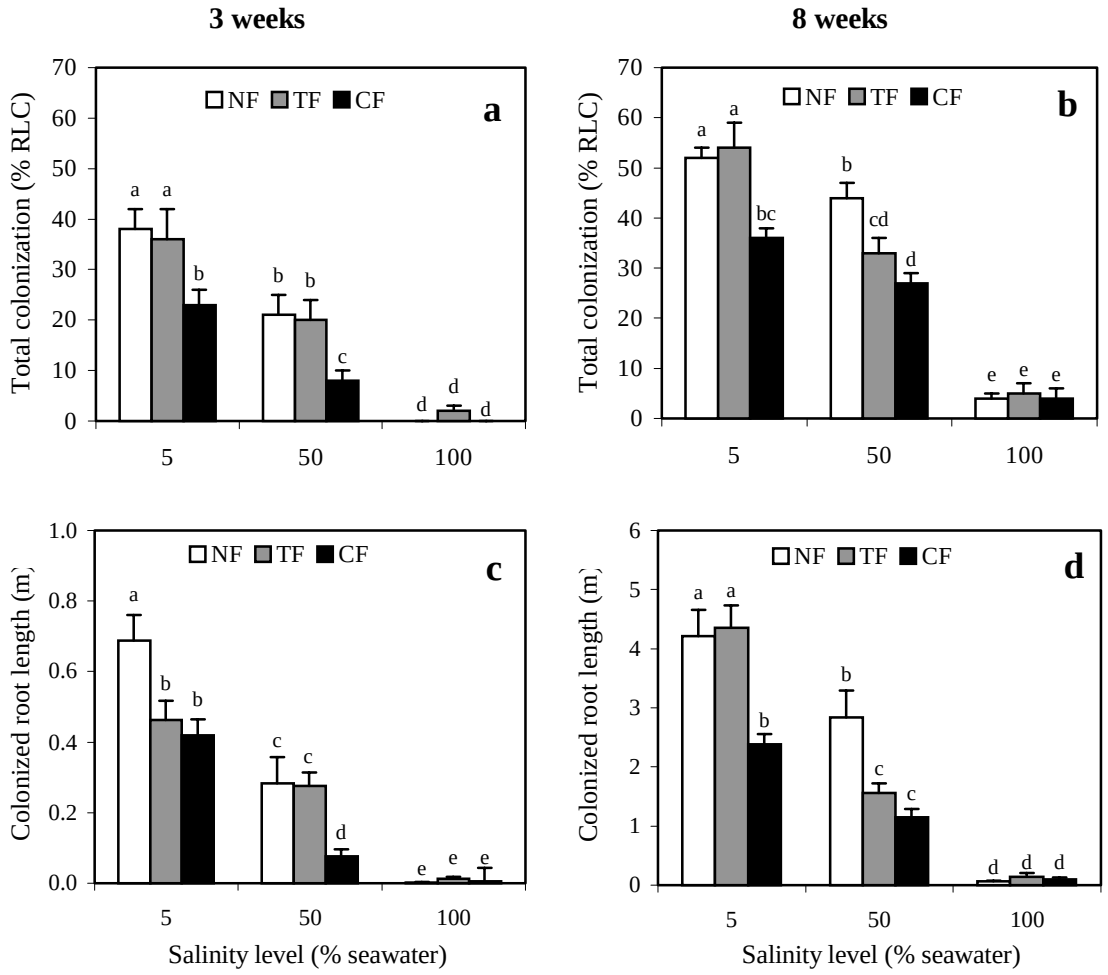

Fig. 1 Total colonization (% root length colonized; *RLC*) (**a**, **b**) and total colonized root length (**c**, **d**) of *Aster tripolium* plants after 3 (**a**, **c**) and 8 weeks (**b**, **d**) of growth under different seawater levels (5%, 50% or 100%) and water regimes [non-flooding (*NF*), tidal flooding (*TF*) or continuous flooding (*CF*)]. In each graph, values (means±SE of six replicates) followed by the *same letter* are not significantly different at $P<0.05$ (Duncan's test)

Percentage of active colonization (measured as metabolic activity) in *A. tripolium* roots was significantly decreased by salinity but unaffected by water regime (Table 1; Fig. 2a). The extent of reduction of active colonization by salinity was proportional to the reduction of total colonization, as showed by the absence of a significant effect of salinity on the proportion of active AMF in roots relative to total AMF in roots (Table 1; Fig. 2b). The proportion of active AMF was affected by water regime (Table 1), showing an increase in the roots of plants exposed to low and intermediate salinity levels (Fig. 2b).

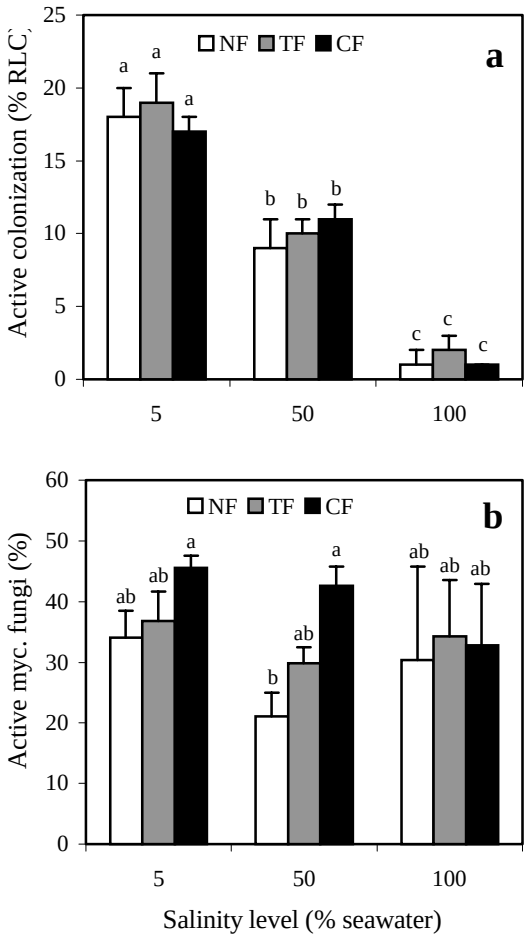

Fig. 2 Metabolically active colonization (%RLC) (**a**) and percentage of mycorrhizal fungi that are metabolically active (**b**) in *A. tripolium* plants after 8 weeks of growth under different seawater levels (5%, 50% or 100%) and water regimes (NF, TF or CF). In each graph, values (means±SE of six replicates) followed by the *same letter* are not significantly different at $P<0.05$ (Duncan's test). For abbreviations, see Fig. 1

ERM length in soil decreased with increasing salinity level and water level, mainly with continuously flooding conditions (decrease of 57%, 70% and 85%, in low, intermediate and high salinity conditions, respectively, relative to non-flooded conditions) (Table 1; Fig. 3a). Salinity and water regime influenced significantly the ratio of root to soil mycelium length (IRM/ERM) (Table 1). The ratio of root to soil mycelium length increased with increasing water level and the highest ratio was found for the intermediate salinity level (Fig. 3b).

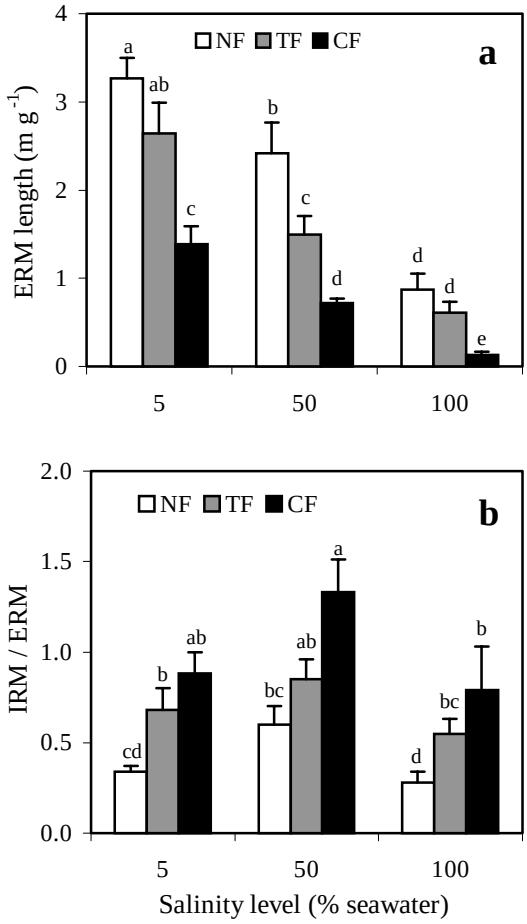

Fig. 3 Length of AM fungal extraradical mycelium *(ERM*) in *A. tripolium* pots (**a**) and ratio of intraradical mycelium length (*IRM*) to ERM (IRM/ERM) (**b**), after 8 weeks of growth under different seawater levels (5%, 50% or 100%) and water regimes (NF, TF or CF). Values (means±SE of six replicates) followed by the *same letter* are not significantly different at $P<0.05$ (Duncan's test). For abbreviations, see Fig. 1

Discussion

The results of this study showed that salinity, flooding and their interaction can influence the initiation and development of AM symbiosis of the salt marsh species *A. tripolium* with indigenous salt marsh AMF, depending on the intensity of the factors. Yet, the activity of AMF was only affected by salinity.

Individual effects of salinity on AM

The ability of salt marsh AMF to colonize *A. tripolium* roots decreased with increasing concentrations of soil salinity, although this did not prevent substantial levels of colonization at a half concentration of seawater. This slight decrease was also reported by Rozema et al. (1986) at similar concentrations, although they used only NaCl as salt and did not test higher concentrations. In our study, the intermediate salinity level influenced more the initiation than the spread of infection, indicating that the fungi are able to expand within the growing root system. Probably the establishment of root colonization of *A. tripolium* in the field may not be greatly dependent on lower and intermediate levels of salinity. In Pancas salt marsh, a negative correlation between field colonization of *A. tripolium* and salinity through the seasons was not found (Carvalho et al. 2001). But, colonization is likely to be almost inhibited if salinity reaches extreme levels, as was observed in our present study. However, the high salt level (full-strength seawater) is not common in Pancas soil (Caçador 1994; Carvalho et al. 2001) but is most typical in salt marshes nearer the Tagus river outfall where neither *A. tripolium* nor AMF have been generally found (personal observation). Indeed, the growth of *A. tripolium* plants was substantially reduced at high levels of salinity and this confirms previous reports (Shennan et al. 1987; Rozema and van Diggelen 1991; Kerstiens et al. 2002), indicating that this species is a moderately salt-tolerant species.

Salinity reduced fungal length and SDH activity. The decline in ERM length suggests that the ability of hyphae to spread in the soil and infect new plants can be affected. Fungal growth and infectivity can be limited, either directly by salt effects (osmotic or ion toxicity), or indirectly through salt effects on plant growth. Salts could affect the capacity of the plant to supply carbohydrates, required for hyphal growth, due to decreased photosynthesis and plant growth with salinity (Munns et al. 1995; McMillen et al. 1998).

Individual effects of flooding on AM

When the water regime was investigated alone the AM colonization was only affected by continuous flooding. In spite of the reduced soil conditions, the incremental increase in flooding level did not prevent the development of infection, with colonization levels similar to those found in the field (Carvalho *et al*. 2001). This indicates that the fungi were able to spread within roots, even under permanently flooded conditions, in agreement with what has been reported for semi-aquatic grasses (Miller and Sharitz 2000).

The reduced growth of *A. tripolium* plants by flooding, as indicated by a decrease in shoot dry weight, may have influenced the growth of AMF. Plant responses to soil oxygen deficiency, such as a decline in photosynthetic capacity, stomatal conductance and nutrient uptake (Kozlowski and Pallardy 1984), may induce a lower allocation of carbohydrates from the host plant to the fungi. A decline in fungal growth due to flooding resulted in a decrease in the non-viable mycelium portions, since activity of AMF was not affected by flooding. This can be explained by the fact that AMF, aerobic microorganisms, acquire the oxygen they need even under reduced soil conditions. Under flooding conditions, *A. tripolium* have the ability to induce a well-developed aerenchyma (Justin and Armstrong 1987), suggesting that AMF could thus overcome the lack of oxygen in soil by colonizing these more oxygenated portions of the root. This could explain the increase in the ratio of root to soil growth of AMF with the increase in water level; thus the effect of flooding on fungal growth was greater in ERM than IRM.

Combined effects of salinity and flooding on AM

The combination of intermediate salinity level and continuous flooding resulted in additive effects on the total extension of colonization and fungal length. However, tidal flooding combined with an intermediate salinity level, which reflected conditions of the salt marsh studied, slightly accentuated the negative effects of salt on total colonization and fungal growth. Also, the effect of the maximum salinity level outweighed any flooding effects, indicating that extreme salinity seems to be more deleterious to *A. tripolium* and/or AM fungal growth than flooding. These results point to the importance of considering salinity and flooding together as joint stressors when studying AM symbiosis in salt marshes.

Conclusion

In conclusion, this study showed that infectivity and activity of salt marsh AMF seems to be mostly dependent on soil salinity rather than on soil flooding. Furthermore, it provides evidence supporting that salt marsh AMF may be particularly adapted to flooding, and their functional role in the symbiosis is likely to be little or not affected since their activity was unaffected by increased flooding. It is therefore possible that AMF may contribute to the establishment of *A. tripolium* along a wider range of soil flooding levels, influencing plant distribution in the salt marsh. However, if these AMF confer flooding tolerance to *A. tripolium* deserves further experimental investigation.

Under scenarios of global change an accelerated sea level rise is expected, changing inundation patterns. This may lead to an inland shift of tidal marshes that become prone to erosion if plants are not able to colonize them. *A. tripolium* is one of the pioneer species present in many European marshes and, as shown in this study, it allows the colonization and expansion of AMF even under high inundation conditions. The possibility of establishing an underground hyphal network linking individuals of this species, may allow a better trapping and stabilization of the sediments brought in by the tide, and consequently, it may contribute to the ability of marshes to keep pace with a sea level rise.

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CAPÍTULO 6

Arbuscular mycorrhiza mitigates growth depression of the salt marsh plant *Aster tripolium* L. caused by tidal flooding

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Arbuscular mycorrhiza mitigates growth depression of the salt marsh plant *Aster tripolium* L. caused by tidal flooding

Luís M. Carvalho[1], Domingos Neto[1], Isabel Caçador[2] and M. Amélia Martins-Loução[1]

[1]Centro de Ecologia e Biologia Vegetal, Departamento de Biologia Vegetal, Faculdade de Ciências de Lisboa, Bloco C2, Campo Grande, 1749-016 Lisboa, Portugal.
[2]Instituto de Oceanografia, Faculdade de Ciências de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal.

Summary

The influence of arbuscular mycorrhizal fungi (AMF) on the growth response of *Aster tripolium* L. plants from two different European salt marshes (Westerschelde, The Netherlands and Tagus, Portugal) to seawater tidal flooding was investigated.
Seedlings of each *A. tripolium* population, either pre-inoculated with AMF present in the salt marsh soil or not inoculated, were subjected over 8 wk to seawater tidal flooding or seawater non-flooding conditions.
AM colonization did not decrease with tidal flooding. The growth of plants from Westerschelde was neither affected by tidal flooding nor improved by mycorrhiza. Tidal flooding considerably reduced the relative growth rate of non-mycorrhizal plants from Tagus, but AM colonization mitigated this reduction through higher net assimilation rate and nitrogen productivity. This positive response was likely linked to a better functional balance between leaves and roots and not directly to phosphorus nutrition.
This study indicates that salt marsh AMF were beneficial to *A. tripolium* growth under seawater tidal flooding improving plant tolerance, though dependent on plant population. It also provides evidence that in salt marshes, the mycorrhizal benefits to plant growth are presumably mediated by environmental factors.

Key words: arbuscular mycorrhiza, *Aster tripolium*, growth analysis, salt marsh, tidal flooding.

Introduction

Salt marshes are harsh environments in which tidal flooding with salt water is known to be a powerful factor influencing the vegetation. The performance, composition and distribution of plant communities are related to the ability of individual species to tolerate the conditions associated with tides, which may vary in frequency and intensity (Armstrong *et al*., 1985; Pennings & Callaway, 1992; Huckle *et al*., 2000). In general, plants from the lower zone of the marsh, more regularly flooded, have to cope with higher water tables, lower redox potential and more stable levels of salinity in the soil, than plants from the higher marsh zone that is irregularly flooded, namely during high tides and precipitation. Besides understanding

anatomical, morphological and physiological plant adaptations to conditions imposed by a regular flooding (Ernst, 1990; Naidoo *et al*., 1992), a broad comprehension of biotic interactions with soil symbionts in the salt marshes is also necessary, even to predict the response of salt marsh plant communities to accelerating sea level rise due to global climate changes, which is thought to affect salt marshes considerably (Adam, 2002).

Association with arbuscular mycorrhizal fungi (AMF) is usually known to enhance plant performance in many ecosystems. Although plant colonization by AMF (e.g. Carvalho *et al*., 2001; Hildebrandt *et al*., 2001) and adaptation of indigenous AMF to salinity and flooding conditions (Carvalho *et al*., 2003a,b) has been found in salt marshes, the ecological function of mycorrhizas in these ecosystems is poorly understood, particularly if the relationship is beneficial to plants under flooding conditions imposed by tidal action. The information about the effects of AMF on plant growth under flooding conditions is scarce and often contradictory. While some studies found negative or no effects of AM colonization (Muthukumar *et al*., 1997; Stevens *et al*., 2002), other studies have shown mycorrhizal benefits in some flooded plant species generally related to an improvement in plant size and phosphorous (P) concentration (Solaiman & Hirata, 1996; Osundina, 1998; Miller & Sharitz 2000). Anderson *et al*. (1986) have suggested that in flooded conditions AM associations may not be functional to the plant, or may even be parasitic. However, all these studies were performed under permanent flooding conditions. To our knowledge, no studies have previously dealt with the influence of AMF on plant performance under salt-water tidal flooding conditions, which is the common water regime of tidal salt marshes.

Aster tripolium L. (sea aster) is a common perennial herb on many European salt marshes with a wide distribution range across the lower and higher zones of the marshes (Huiskes *et al*., 1985), though being a moderately salt tolerant species (Shennan *et al*., 1987; Kerstiens *et al*., 2002). *A. tripolium* plants are commonly colonized by AMF in the field (Rozema *et al*., 1986; Carvalho *et al*., 2001; Hildebrandt *et al*., 2001) and colonization levels did not differ between less and more flooded marsh zones (Carvalho *et al*., 2001). A previous experiment showed that salt marsh AMF are able to initiate and promote colonization in *A. tripolium* roots under seawater tidal flooding conditions present in the salt marsh (Carvalho *et al*., 2003a). Moreover, AM fungal activity was not affected by tidal or permanent flooding conditions, suggesting that AMF may have the potential to provide benefits to *A. tripolium* under those conditions.

This work aimed to investigate whether salt marsh AMF improve *A. tripolium* tolerance to seawater tidal flooding. Specifically, we tested the hypotheses that (i) mycorrhizal plants have

higher growth performance than non-mycorrhizal plants under tidal flooding conditions and (ii) mycorrhizal benefits to plant growth are independent of watering conditions (non-flooding and tidal flooding). We performed growth analysis to evaluate whether AMF influence the functional balance between leaves and roots either in terms of mass allocation and morphology as in terms of physiological activity (Wright & Westoby, 2000). Two populations of *A. tripolium* from two different European salt marshes (Dutch and Portuguese) were used to assess whether the response patterns of *A. tripolium* to tidal flooding and AMF are plant population-dependent. These populations are morphologically distinct, presenting the Dutch population wider and longer leaves, lower shoot height and lower root length than the Portuguese one (pers. observ.). In fact, genotypic and environmental variation has been found in *A. tripolium* of different salt marshes (Huiskes *et al*., 2000).

Materials and Methods

Soil and mycorrhizal inoculum

The soil used as mycorrhizal inoculum was collected within *A. tripolium* stands in the Pancas salt marsh (Tagus estuary, Portugal), in order to obtain mycorrhizal plants that were colonized by a natural population of AMF representative of those present in the rizosphere of *A. tripolium*. The soil had a pH (H_20) of 6.0, 11.9% organic matter, 2 g total N kg^{-1}, 900 mg extractable P kg^{-1} and 6 spores g^{-1} dry soil comprising only *Glomus* species, with *Glomus geosporum* (Nicol. and Gerd.) Walker as the most abundant species (Carvalho *et al*., 2001, 2003c). The washed river sand (<1 mm) used in this experiment was autoclaved at 110ºC for 1 h on two consecutive days and allowed to stabilize for 2 wk.

Plant material

Sterilized seeds of *A. tripolium* collected in autumn from Waarde salt marsh, Westerschelde estuary, The Netherlands (Westerschelde population), and from Pancas salt marsh, Tagus estuary, Portugal (Tagus population) were germinated on Petri dishes lined with moistened filter paper and placed in the dark at 25ºC for 7-10 d. Uniform seedlings were then transferred to plastic containers ($40 \times 28 \times 7.5$ cm^3) containing autoclaved sand. Potted seedlings were grown for 4 wk in a controlled environmental growth room, with temperatures of 25 / 18ºC (day/night), a photoperiod of 14 h at a quantum flux density of 400 μmol m^{-2} s^{-1} and 50%

relative humidity. The plants were watered frequently with tap water and received 1/4-strength Hoagland modified solution (Hoagland & Arnon, 1939) with a reduced P concentration (0.1 mM) once every 2 wk.

Mycorrhizal inoculation

After 4 wk, 25 plants of each population were individually transplanted to 250-ml plastic pots (8 cm diameter) filled with a 3 : 1 (v/v) mixture of autoclaved sand and non-sterile (AM plants) or sterile (non-mycorrhizal, NM plants) salt marsh soil. A filtrate obtained from the soil that was passed through a filter (Whatman no. 42) was added to each NM pot in order to reintroduce the soil microbial community (excluding the AMF) associated with *A. tripolium* roots. The plants grew in the same controlled environmental growth room described above in a completely randomised design. The pots were randomly rotated at weekly intervals to avoid site effects within the growth room. The plants were watered alternately with tap water and artificial seawater solution 50% diluted (adapted from Epstein, 1972) representing salt marsh conditions (Carvalho *et al*., 2003a). To avoid osmotic shock, it was applied 25% seawater concentration at the beginning and it was only increased to 50% after 2 d. The plants were allowed to grow for 8 wk to ensure that substantial levels of colonization were present in the AM plants from the start of the imposition of water regime treatments.

Experimental design

After the inoculation period, a $2 \times 2 \times 2$ factorial experimental design in a split-plot with two water regimes (non-flooding and tidal flooding) as the main plot, and with two mycorrhizal treatments (AM and NM) and two *A. tripolium* populations (Westerschelde and Tagus origins) as subplots, was established. There were five replicates assigned to each treatment.

AM and NM plants with the respective soil substrate (see above) were individually transplanted to larger plastic pots (1250 ml, 10 cm diameter). Each pot contained expanded clay at the bottom, sand and the transferred substrate (1 : 3 : 1, v/v/v). The pots were randomly assigned to ten containers belonging each half to each of the two types of water regime. Each container comprised pots representing the four pairwise combinations of *A. tripolium* populations and mycorrhizal inoculation treatments. The non-flooded pots were watered daily to field capacity with water or artificial seawater solution 50% diluted as necessary. In the tidally flooded treatments a system device was set up to simulate a tidal regime of 1 h of flooding at 20-h intervals. A pump connected to a programmed timer was

used to transfer artificial seawater solution 50% diluted from tanks to the containers (each tank was assigned to one container), simulating tidal action. A 2-cm inundation level above the pots was maintained during flooding (a small hole in the containers released the excess of water). Tidal flooding treatments had lower redox potentials than non-flooded treatments (Carvalho *et al.*, 2003a). Water was added periodically to tanks in order to replace evapotranspirational losses. Salinity was monitored regularly with a hand-held refractometer and seawater solutions were renewed every wk. A 1/4-strength Hoagland solution with a reduced P concentration (0.1 mM) was added to all pots once every 2 wk. Plants grew under greenhouse conditions with midday light intensity (PAR) inside the greenhouse of 500 μmol m^{-2} s^{-1} at plant level, averages of minimal and maximal temperatures were 18ºC and 28ºC, respectively, and relative humidity was around 50%.

Harvest and material analysis

On the first day water regimes were imposed (end of inoculation period), 5 plants of each *A. tripolium* population and mycorrhizal treatment combination were harvested (initial harvest). After 8 wk of growth under non-flooding or tidal flooding conditions, 5 plants per treatment were harvested (final harvest). Plant material were separated into leaves, stem and roots, cleaned and fresh weights were determined. Roots and leaves were immediately scanned for total root length and root surface area, and total leaf area determination, respectively, by digital image analysis with WinRhizo software (Regent Instruments Image Analysis Systems, Canada). A 0.3-0.5 g (fresh weight) subsample was removed from each root system and stored in 50% ethanol for later determination of AM colonization. Dry weights of the remaining roots and of the other plant fractions were determined after drying at 60ºC for 5 d. The dry weight : fresh weight ratio of the remaining roots was used to calculate the dry weight of the root subsample and then of the entire root sample. Nitrogen (N) concentration was determined using a CHNS elemental analyzer (EuroVector EA, Italy) and P concentration by the molybdenum blue method (Murphy & Riley, 1962).

Roots were cleared and stained for analysis of total colonization by AMF through a modified Phillips & Hayman (1970) procedure (Carvalho *et al.*, 2001). Root colonization was estimated by the gridline intersection method at ×45-190 magnification (Giovannetti & Mosse, 1980) and expressed as percentage of root length colonized and colonized root length (m). Two soil cores were also taken from each pot at the final harvest for the quantification of the extraradical mycelium (ERM) length. The two soil cores were mixed and 5 g fresh weight

was dried (60ºC, 5 d) for dry weight determination and another 5 g fresh weight was used to extract ERM from the soil by a modified Miller *et al*. (1995) procedure (Carvalho *et al*., 2003b). ERM length was estimated by the gridline intersect method by examining 140 fields of view at ×200 magnification. Only aseptate hyphae with a characteristic 'knobby' appearance and dichotomous branching were considered as AM fungal hyphae. Total hyphal length was calculated based on Tennant (1975) and converted to meters per g dry soil.

Data analysis

Plant growth were examined by calculating relative growth rate (RGR), which takes into account the differences between AM and NM plants at the initial harvest. RGR can be factorized into the product of the physiological component net assimilation rate (NAR), also known as unit leaf rate (ULR), and the morphological component leaf area ratio (LAR, the ratio of leaf area to whole-plant dry mass) (Evans, 1972). RGR and NAR were calculated between the two harvests (i, initial, and f, final) according to Hunt (1978):

$$RGR = (\ln W_f - \ln W_i) / t_f - t_i \quad \text{Eqn 1}$$

$$NAR = ((W_f - W_i) / (t_f - t_i)) \times ((\ln LA_f - \ln LA_i) / (LA_f - LA_i)) \quad \text{Eqn 2}$$

where W is the whole-plant dry mass, LA is leaf area, t is time and mean were used for the initial harvest. LAR is the product of specific leaf area (SLA, the ratio of leaf area to leaf dry mass) and leaf mass fraction (LMF, the fraction of leaf dry mass to whole-plant dry mass). Root mass fraction (RMF, the fraction of root dry mass to whole-plant dry mass), specific root length (SRL, the ratio of total root length to root dry mass), root length ratio (RLR, the ratio of total root length to plant dry mass) and leaf area to root length ratio were also calculated.

From a N perspective, RGR can be factorized into the product of leaf N productivity (LNP, the rate of plant dry mass produced per unit of leaf N and time), leaf N concentration (LN) and LMF (Wright & Westoby, 2000). LNP was determined as:

$$LNP = ((W_f - W_i) / (t_f - t_i)) \times ((\ln LN_f - \ln LN_i) / (LN_f - LN_i)) \quad \text{Eqn 3}$$

N uptake intensity was determined as net N absorption rate (NNAR, the rate of increase in plant N per unit RSA and per time) according to Lutze & Gifford (1998):

$$NNAR = ((NP_f - NP_i) / (t_f - t_i)) \times ((\ln RSA_f - \ln RSA_i) / (RSA_f - RSA_i)) \quad \text{Eqn 4}$$

where RSA is root surface area.

Plant host benefit was expressed as the percentage change in AM plant response compared to NM plants. Plant benefit was calculated as reported by Gange & Ayers (1999):

Plant benefit (%) = ((m - n) / n) × 100 Eqn 5

where m is the average of RGR of the AM plants and n the average of RGR of the NM plants, grown in the same water regime conditions.

Statistical analysis

Data were analyzed using the GLM procedure (SPSS 11.5, Chicago, IL, USA). Data from the initial harvest were analyzed with a two-way ANOVA to test effects of mycorrhizal inoculation and *A. tripolium* population. Data from the final harvest were analyzed with a three-way ANOVA for split-plot design to assess effects of water regime, mycorrhizal inoculation and *A. tripolium* population (fixed factors). Container was the experimental unit and was included in the analysis as a random factor. In the percentage of root length colonized and colonized root length variables, the non-mycorrhizal treatments (the results were null) were entirely removed from the analysis and a one-way or a two-way ANOVA, for initial or final harvest, respectively, was used. Significant results (P<0.05) were analysed by Duncan's test. Prior to statistical analysis, data were log-transformed as necessary to satisfy the assumptions of the ANOVA (Zar, 1984). To determine whether apparent differences in biomass allocation and formation of leaf *vs* root surface were due to direct treatment effects or to treatment-mediated differences in overall plant size (Hunt, 1978; Coleman *et al*., 1994), LMF, RMF and leaf area to root length ratio were also analysed by ANCOVA, using log-transformed plant dry mass as the covariate (Poorter & Nagel, 2000).

Results

Mycorrhizal inoculation period (initial harvest)

High levels of root colonization by AMF were found in the AM plants harvested before water regimes were applied (Fig. 1). The colonization was significantly higher in the Tagus population ($F_{1,8}$ = 18.29, P < 0.01 for percentage of root length colonized and $F_{1,8}$ = 23.67, P < 0.01 for colonized root length). All NM plants were not colonized. Mycorrhizal inoculation showed a negative effect on plant dry mass regardless of plant population (Fig. 2; $F_{1,16}$ = 24.57, P < 0.001 for mycorrhiza main effect, $F_{1,16}$ = 0.001, P > 0.05 for population main effect and $F_{1,16}$ = 0.65, P > 0.05 for interaction effect). In each plant population, mycorrhizal

inoculation significantly decreased leaf area and had no significant effects ($P > 0.05$) on LAR, SLA, LMF and RMF (data not shown).

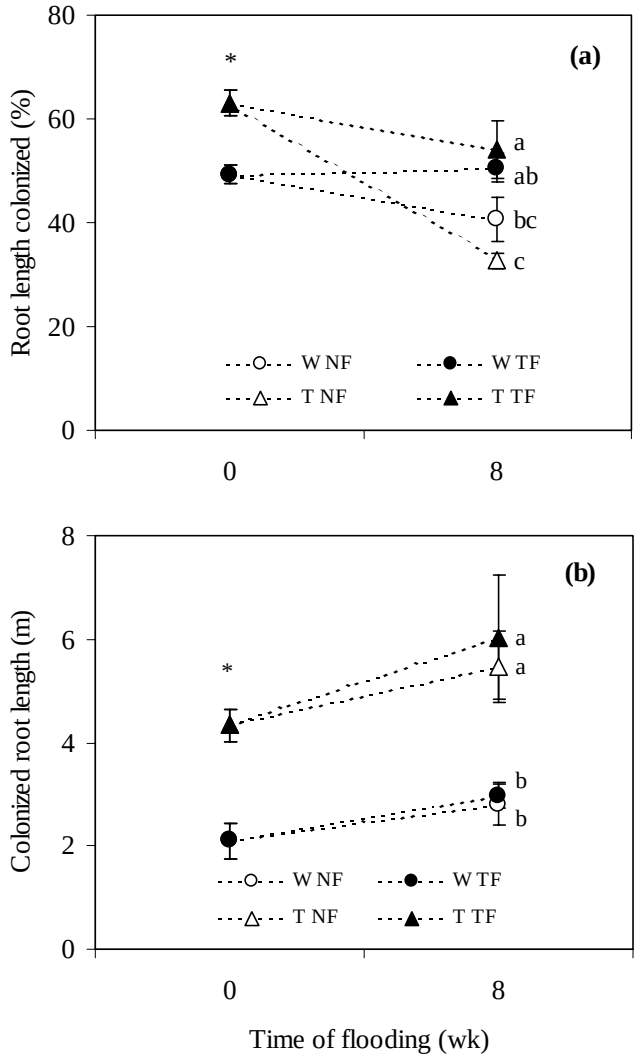

Fig 1 Percentage of root length colonized (a) and colonized root length (b) of mycorrhizal *A. tripolium* plants from Westerschelde (W) and Tagus (T) populations after 0 (start) and 8 wk of growth under different water regimes (non-flooding, NF or tidal flooding, TF). Values are means ± SE of 5 replicates. In each graph, asterisk indicates significant difference between populations at week 0 and different letters indicate significant differences between treatments at week 8 ($P < 0.05$, Duncan's test).

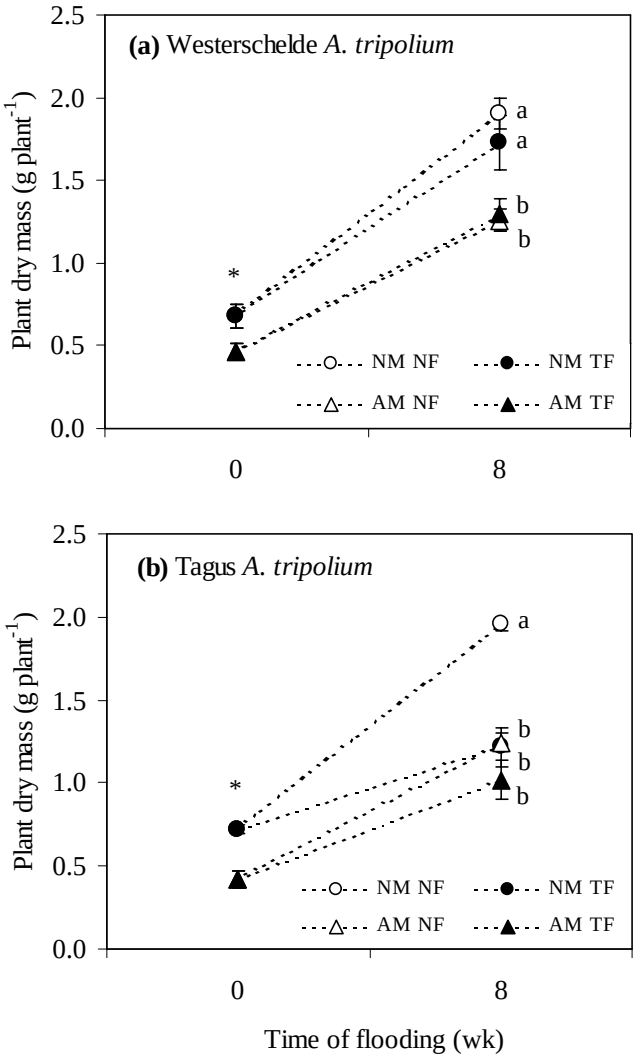

Fig 2 Plant dry mass of non-mycorrhizal (NM) and mycorrhizal (AM) *A. tripolium* plants from Westerschelde (a) and Tagus (b) populations after 0 (start) and 8 wk of growth under different water regimes (non-flooding, NF or tidal flooding, TF). Values are means ± SE of 5 replicates. In each graph, asterisk indicates significant difference between populations at week 0 and different letters indicate significant differences between treatments at week 8 ($P < 0.05$, Duncan's test).

Tidal flooding effects (final harvest)

At the end of the experiment, water regime significantly influenced percentage of root length colonized ($F_{1,8} = 13.22$, $P < 0.01$), but it was dependent on plant population ($F_{1,8} = 1.65$, $P < 0.05$ for interaction effect and $F_{1,8} = 0.98$, $P > 0.05$ for population main effect). A significant increase in percentage of root length colonized with tidal flooding was only found for plants of the Tagus population (Fig. 1a). However, this increase was not reflected in the colonized root length (Fig. 1b), showing that the difference was not due to lower fungal growth. Both populations differed in the colonized root length ($F_{1,8} = 15.24$, $P < 0.01$), but water regime ($F_{1,8} = 0.20$, $P > 0.05$) and interaction between both factors had no effect ($F_{1,8} = 0.04$, $P >$

0.05). Colonization was not observed in the NM plants. Tidal flooding ($F_{1,8}$ = 4.10, P > 0.05) and *A. tripolium* population ($F_{1,8}$ = 0.68, P > 0.05) did not significantly affect ERM length of AMF ($F_{1,8}$ = 1.32, P > 0.05 for interaction effect), although there was a trend to a decrease with tidal flooding, in particular in the Tagus plants (3.62 m g^{-1} ± 0.40 SE and 2.61 m g^{-1} ± 0.32 for Tagus NF and TF, respectively; 2.69 m g^{-1} ± 0.52 and 2.40 m g^{-1} ± 0.37 for Westerschelde NF and TF, respectively).

Water regime ($F_{1,8}$ = 20.04, P < 0.01), mycorrhizal inoculation ($F_{1,24}$ = 44.90, P < 0.001) and plant population ($F_{1,24}$ = 6.33, P < 0.05) influenced plant dry mass, which was also affected by the interactions of water regime with mycorrhizal inoculation ($F_{1,24}$ = 5.90, P < 0.05) and with plant population ($F_{1,24}$ = 7.78, P < 0.05). In the Westerschelde population, plant dry mass was not affected by tidal flooding and was higher in the NM plants than in the AM plants (Fig. 2a). In the Tagus population, tidal flooding only affected dry mass in the NM plants (62% of reduction compared to non-flooding conditions) and the highest dry mass was found in the NM plants under non-flooding conditions (Fig. 2b).

Table 1 Plant growth parameters (relative growth rate, RGR; net assimilation rate, NAR; leaf area ratio, LAR; specific leaf area, SLA; leaf mass fraction, LMF; root mass fraction, RMF) of non-mycorrhizal (NM) and mycorrhizal (AM) *A. tripolium* plants from Westerschelde and Tagus populations after 8 wk of growth under different water regimes (non-flooding, NF or tidal flooding, TF). Values (means ± SE of 5 replicates) and summary of ANOVA of the effects of water regime (W), mycorrhizal inoculation (M) and *A. tripolium* population (P) on those plant parameters are shown. Values within each column and within each population followed by the same letter are not significantly different at P < 0.05 (Duncan's test)

A. tripolium population	AM status	Water regime	RGR (mg g^{-1} d^{-1})	NAR (g m^{-2} d^{-1})	LAR (m^2 kg^{-1})	SLA (m^2 kg^{-1})	LMF (g g^{-1})	RMF (g g^{-1})
Westerschelde	NM	NF	18.3 ± 0.9a	4.11 ± 0.30a	3.38 ± 0.28b	7.1 ± 1.0a	0.49 ± 0.03a	0.36 ± 0.03b
		TF	16.4 ± 1.9a	4.00 ± 0.52a	2.80 ± 0.07c	7.1 ± 0.5a	0.40 ± 0.02b	0.45 ± 0.03a
	AM	NF	17.7 ± 0.9a	3.29 ± 0.22a	4.01 ± 0.22a	8.1 ± 0.5a	0.50 ± 0.01a	0.37 ± 0.00b
		TF	18.0 ± 1.5a	4.07 ± 0.34a	2.70 ± 0.06c	6.7 ± 0.1a	0.41 ± 0.01b	0.43 ± 0.02ab
Tagus	NM	NF	17.8 ± 0.4a	3.30 ± 0.15a	3.99 ± 0.23a	10.9 ± 0.4a	0.37 ± 0.01ab	0.51 ± 0.01ab
		TF	9.1 ± 1.6b	2.20 ± 0.32b	2.00 ± 0.24c	7.7 ± 0.6b	0.26 ± 0.02c	0.57 ± 0.02a
	AM	NF	19.2 ± 0.8a	3.31 ± 0.25a	4.51 ± 0.31a	10.9 ± 0.3a	0.41 ± 0.02a	0.48 ± 0.02b
		TF	16.9 ± 1.6a	3.60 ± 0.37a	2.98 ± 0.10b	8.6 ± 0.2b	0.35 ± 0.02b	0.48 ± 0.03b
Source of variation		df	*F*-values					
W		1, 8	15.85**	0.01	60.24***	16.95**	87.44***	26.21**
M		1, 24	7.82*	0.57	13.75**	1.25	6.93*	3.28
P		1, 24	3.70	10.87**	1.06	50.12***	54.23***	32.58***
W × M		1, 24	5.74*	6.38*	0.21	0.11	0.37	1.28
W × P		1, 24	5.90*	2.52	8.87**	9.30**	0.01	1.24
M × P		1, 24	4.84*	5.76*	3.14	0.08	4.62*	1.78
W × M × P		1, 24	1.43	0.33	4.88*	2.92	0.72	0.05

* P < 0.05; ** P < 0.01; *** P < 0.001.

Tidal flooding affected the RGR of plant dry mass, but the effects were dependent on the mycorrhizal treatment and plant population, as indicated by the significant interactions of each one of these factors with water regime (Table 1). The RGR of the Westerschelde plants was neither affected by mycorrhizal treatment nor by tidal flooding (Table 1). In the Tagus population, NM plants under tidal flooding showed the lowest RGR, whereas AM plants were not affected by the increase in the water level (Table 1). Westerschelde *A. tripolium* plants showed no benefit in their RGR from the association with AMF under non-flooding (-3.1%) and a small benefit under tidal flooding conditions (10.4%), while Tagus *A. tripolium* plants have a very high benefit under tidal flooding (87.1%) and a small one under non-flooding conditions (7.4%).

Tidal flooding decreased RGR of the Tagus NM plants through reductions in NAR and LAR, which was 37% and 50%, respectively, lower than in the non-flooded plants (Table 1). In the Tagus AM plants, tidal flooding did not significantly affect NAR and decreased LAR, although this decrease did not result in variation of RGR. NAR of the Westerschelde plants was unaffected by treatments and LAR decreased with tidal flooding, but no variation was observed in the RGR of these plants (Table 1). Mycorrhizal inoculation *per se* generally enhanced LAR. The decrease of LAR by tidal flooding in the Westerschelde plants was only accounted by decreases in LMR, while in the Tagus plants the tidal flooding decreased both SLA and LMF (more pronounced in the NM plants) (Table 1). Mycorrhizal inoculation had no effect on SLA and significantly enhanced the LMF of the Tagus plants, namely under tidal flooding conditions, which led to higher LAR compared to NM conditions. Water regime and plant population significantly affected RMF (Table 1). There was a trend to increase RMF with tidal flooding except in the Tagus AM plants. The treatments effects on LMF and RMF remained when plant dry mass was incorporated in the model as a covariate ($P > 0.05$), indicating that the differences were due to treatments *per se* and not to plant size differences. The correlation between LMF or RMF and plant dry mass were not significant ($n = 40$, $r = 0.28$, $P > 0.05$ for LMF and $n = 40$, $r = -0.17$, $P > 0.05$ for RMF).

Final leaf area was negatively affected by tidal flooding, in particular in the Tagus plants (Table 2). AM colonization decreased leaf area, except in the Tagus tidal flooded plants. Although the total leaf number significantly decreased with tidal flooding, the abscission of leaves over the experiment was not significantly affected by water regime (data not shown). Roots of Tagus plants were much longer and had a higher SRL and RLR than Westerschelde plant roots (Table 2). Tidal flooding and AM colonization decreased root length, in particular in the Tagus plants. Treatment effects on root surface area were similar to those on root length

(data not shown). Tidal flooding affected SRL, namely in the Westerschelde plants, whereas AM colonization had no significant effects. In the Tagus population, the AM plants had lower RLR than the AM plants at tidal flooded conditions (Table 2). Tidal flooding decreased leaf area : root length ratio, although it was only significant in the Tagus NM plants, while AM colonization increased that ratio, in particular in the Tagus tidal flooded plants (Table 2). The treatments effects on leaf area : root length ratio remained when plant dry mass was incorporated in the model as a covariate ($P > 0.05$), indicating that the differences were due to treatments *per se* and not to plant size differences. The correlation between leaf area : root length ratio and plant dry mass were not significant ($n = 40$, $r = 0.25$, $P > 0.05$).

Table 2 Leaf area (LA), root length (RL), specific root length (SRL), root length ratio (RLR), leaf area : root length ratio (LA : RL) of non-mycorrhizal (NM) and mycorrhizal (AM) *A. tripolium* plants from Westerschelde and Tagus populations after 8 wk of growth under different water regimes (non-flooding, NF or tidal flooding, TF). Values (means ± SE of 5 replicates) and summary of ANOVA of the effects of water regime (W), mycorrhizal inoculation (M) and *A. tripolium* population (P) on those plant parameters are shown. Values within each column and within each population followed by the same letter are not significantly different at $P < 0.05$ (Duncan's test)

A. tripolium population	AM status	Water regime	LA (cm^2 $plant^{-1}$)	RL (m $plant^{-1}$)	SRL (m g^{-1})	RLR (m g^{-1})	LA : RL (m^2 m^{-1})
Westerschelde	NM	NF	63.7 ± 4.1a	12.0 ± 0.9a	17.8 ± 2.0a	6.4 ± 0.6a	5.52 ± 0.68b
		TF	48.1 ± 3.7b	9.5 ± 1.1b	12.4 ± 0.1b	5.5 ± 0.3ab	5.14 ± 0.33b
	AM	NF	50.3 ± 2.8b	6.8 ± 0.4c	14.7 ± 1.1ab	5.5 ± 0.4ab	7.43 ± 0.42a
		TF	35.0 ± 3.2c	5.9 ± 0.3c	11.1 ± 1.5b	4.6 ± 0.3b	5.95 ± 0.38ab
Tagus	NM	NF	78.0 ± 4.4a	25.9 ± 0.8a	26.2 ± 1.6a	13.3 ± 0.7ab	3.02 ± 0.14a
		TF	25.0 ± 4.9c	18.6 ± 2.2b	27.9 ± 3.9a	15.6 ± 1.9a	1.35 ± 0.23b
	AM	NF	55.6 ± 3.1b	16.8 ± 2.2b	27.9 ± 1.8a	13.4 ± 1.4ab	3.65 ± 0.70a
		TF	30.2 ± 3.5c	9.8 ± 1.4c	20.4 ± 2.5a	10.0 ± 0.3b	3.48 ± 0.89a
Source of variation		df	*F*-values				
W		1, 8	164.07***	26.72**	18.28**	3.14	27.06***
M		1, 24	14.95**	47.60***	3.02	6.74*	14.60***
P		1, 24	0.54	80.04***	58.15***	117.30***	73.14***
W × M		1, 24	6.12*	0.11	1.03	2.56	2.29
W × P		1, 24	17.76***	2.77	1.14	0.11	2.85
M × P		1, 24	0.65	0.11	0.03	0.25	2.03
W × M × P		1, 24	5.84*	0.82	1.70	1.87	5.87*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Tidal flooding and AM colonization had no significant effect on leaf P concentration regardless the watering conditions and plant population (Table 3). Overall, AM plants had higher leaf N concentrations compared to NM plants for both populations (Table 3). AM colonization improved NNAR under tidal flooding conditions, which had a negative effect on NNAR of the NM plants (Table 3). Increased N concentration due to AM inoculation was not

fully expressed in leaf N productivity except for the Tagus AM plants under tidal flooding conditions (Table 3).

Table 3 Leaf concentration of phosphorous (P) and nitrogen (N), net N absorption rate (NNAR) and leaf N productivity (LNP) of non-mycorrhizal (NM) and mycorrhizal (AM) *A. tripolium* plants from Westerschelde and Tagus populations after 8 wk of growth under different water regimes (non-flooding, NF or tidal flooding, TF). Values (means ± SE of 5 replicates) and summary of ANOVA of the effects of water regime (W), mycorrhizal inoculation (M) and *A. tripolium* population (P) on those plant parameters are shown. Values within each column and within each population followed by the same letter are not significantly different at $P < 0.05$ (Duncan's test)

A. tripolium population	AM status	Water regime	Leaf P (mg g^{-1})	Leaf N (mg g^{-1})	NNAR (mg m^{-2} d^{-1})	LNP (g g^{-1} d^{-1})
Westerschelde	NM	NF	0.41 ± 0.04a	10.6 ± 0.9ab	10.6 ± 2.5a	2.84 ± 0.15a
		TF	0.38 ± 0.03a	8.3 ± 1.3b	3.0 ± 0.9b	3.31 ± 0.54a
	AM	NF	0.36 ± 0.03a	13.3 ± 1.9a	17.3 ± 3.4a	2.26 ± 0.21a
		TF	0.37 ± 0.03a	13.1 ± 0.6a	12.5 ± 1.7a	2.50 ± 0.21a
Tagus	NM	NF	0.49 ± 0.02a	11.0 ± 0.3bc	6.5 ± 0.6b	3.82 ± 0.15a
		TF	0.50 ± 0.06a	10.2 ± 0.4c	1.8 ± 0.5c	2.35 ± 0.39b
	AM	NF	0.48 ± 0.04a	13.2 ± 0.4a	11.4 ± 0.9a	3.42 ± 0.22a
		TF	0.50 ± 0.06a	12.2 ± 1.1ab	6.9 ± 0.6b	3.73 ± 0.58a
Source of variation		df	*F*-values			
W		1, 8	0.04	2.31	29.65**	0.13
M		1, 24	0.40	18.57***	50.24***	0.18
P		1, 24	12.58**	0.57	9.77**	7.84*
W × M		1, 24	0.19	0.80	8.00*	3.30
W × P		1, 24	0.19	0.04	0.11	4.49*
M × P		1, 24	0.11	1.83	0.18	7.62*
W × M × P		1, 24	0.14	1.09	0.20	5.53*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Discussion

The results of this work showed that the two studied *A. tripolium* populations differed in their growth response to seawater tidal flooding and AM colonization. Tidal flooding did not affect the growth of plants from the Westerschelde population. Plant growth depression of the Tagus population caused by tidal flooding was mitigated by the presence of AM association, supporting, for this population, our first hypothesis that AM plants had higher growth performance than NM plants. Contrarily to our second hypothesis, AM beneficial effects on plant growth were dependent on the watering conditions. There was no evidence of AM benefits under non-flooding conditions.

Before water regime treatments were applied, mycorrhizal inoculation resulted in plants with lower biomass. This result can be associated with AM detrimental effects in the initial

phases of plant growth (Johnson *et al*., 1997) or to growth limitation of roots and hyphae due to pot size. The reduced size of AM plants did not conditioned afterwards plant growth rate, since at the final harvest the AM plants had equal or higher RGR compared to NM plants.

Our results also support previous findings indicating indigenous AM fungal tolerance to the salinity and flooding conditions present in the Pancas salt marsh (Carvalho *et al*., 2003a,b), since once colonization is established, the maintenance and expansion of the AM association and AMF growth were not susceptible to tidal activity. Miller & Sharitz (2000) reported similar results in semiaquatic grasses under non-salt permanent flooding conditions.

Tidal flooding influenced mainly the leaf traits of *A. tripolium* plants. Morphologically, both populations responded to tidal flooding having lower leaf area and allocating less biomass to leaves and more to roots. From a whole-plant perspective, these changes induced a reduction in LAR, which was only accompanied by a decrease in RGR in the Tagus NM plants due to a high reduction in the physiological component (NAR) in these plants. The higher tolerance of the Westerschelde plants to tidal flooding compared to Tagus NM plants, was likely due to higher physiological performance that probably counterbalanced the observed reductions in the morphological traits. Flooding tolerance of *A. tripolium* from a Dutch salt marsh has been previously described (Lenssen *et al*., 1995). In that work, permanent-flooding conditions with salt water had no effects in the photosynthetic rate, one of the components of NAR (Lambers & Poorter, 1992), and decreased LAR without affecting whole-plant growth.

In the Tagus population, the higher RGR of the AM plants compared to NM plants was associated with a higher NAR and LAR. The increase of the latter was due to a shift in biomass partitioning towards leaves. Miller & Sharitz (2000) found that the growth improvement of semiaquatic grasses by mycorrhizas under flooding conditions was accompanied by an improvement of P nutrition. In the present study, the beneficial effect of AMF to Tagus flooded plants cannot be attributed to an improvement of P nutrition, because AM and NM plants had similar P concentrations as it was observed in a previous report for *A. tripolium* under permanent flooding conditions (Rozema *et al*., 1986). Nevertheless, our study showed an improvement effect of AMF on N uptake intensity and N concentration. However, from a N perspective, the results showed that changes in RGR of *A. tripolium* were mainly associated with variations in N productivity than with changes in N concentration. Thus, differences in growth between NM and AM plants of the Tagus population may reflect differences in how ‘efficiently’ plant N was used in the growth process. In herbs the rate of photosynthesis per unit leaf N is a major determinant of N productivity (Garnier *et al*., 1995),

reflecting the investment of N in photosynthetic and non-photosynthetic leaf components (Lambers & Poorter, 1992).

AMF are known to influence root morphology (Fitter, 1977). In our study, root fineness (SRL) was not influenced by AM colonization but root length decreased, particularly in the Tagus plants under flooding conditions. From a whole-plant perspective, this change resulted in lower root length per C invested in plant mass (RLR). The plant probably allocates less energy to root production since the hyphae enhance the nutrient absorbing surface in the soil. The better performance of Tagus AM plants under flooding conditions was unlikely due to a lower demand of C to the AMF, since a previous study showed that the activity of indigenous AMF from Pancas salt marsh in *A. tripolium* plants was not affected by flooding conditions (Carvalho *et al*., 2003a). In these AM plants, the higher allocation of biomass to leaves than to roots, coupled with a decrease in the root length without reducing nutrient acquisition, led to an improvement of the functional balance between leaves and roots activities compared to NM plants. This may have led to an increase in the net result of C gain (photosynthetic rate measured in all leaves and integrated over the day) and C losses (shoot and root respiration, including that of the mycorrhizal fungi, exudation, and volatilization) expressed per unit leaf area, i.e., the NAR (Lambers & Poorter, 1992), and to a higher N productivity. We, therefore, propose that the maximization of the functional balance between leaves and roots (total plant capacity for photosynthetic C gain *vs* mineral acquisition intensity) together with a more efficient use of N taken up are important factors explaining the role of AMF on *A. tripolium* tolerance to tidal flooding.

This present study indicates that mycorrhizal benefits in *A. tripolium* growth are likely mediated by abiotic factors and dependent on plant population or on plant tolerance ability to those factors. The apparent absence of a beneficial response in the growth of the Westerschelde plants to AMF does not necessarily imply that in the field, a more complex system where plants of this population are well colonized (L.M. Carvalho *et al*., unpublished), the outcome of the interaction would not be different. Furthermore, benefits of the association may only exceed costs to the plants in later phases of the plant life cycle, such as in the reproductive stage (Johnson *et al*., 1997). Other possible explanation for the lack of plant benefits is that the association between the Westerschelde plants and AMF from the Tagus site are not effective to the plant, since mycorrhizal responsiveness can be dependent on the origin of the plant and the fungal population (Ronsheim & Anderson, 2001; van der Heijden & Kuyper, 2001).

In conclusion, this study showed that AMF improve *A. tripolium* tolerance to seawater tidal flooding, at least in plants from the Tagus population, ameliorating plant growth. Higher tolerance induced by AMF may allow *A. tripolium* plants to colonize successfully more flooded zones of the salt marshes and may enhance their ability to withstand the increase of flooding due to the accelerating sea level rise.

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CAPÍTULO 7

Discussão geral

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Este trabalho permitiu adquirir novos conhecimentos relativos à ocorrência e distribuição das AM em sapais, à adaptação e infectividade dos fungos AM sob condições abióticas presentes nos sapais e aos benefícios das AM para as plantas de sapal, contribuindo de modo significativo para aumentar a compreensão da ecologia das micorrizas neste tipo de ecossistema, particularmente adverso para os organismos vivos. Os resultados deste trabalho demonstraram também que os atributos ecológicos dos fungos AM nos sapais são regulados pela interacção entre factores bióticos (tipo e características das plantas) e abióticos (salinidade e alagamento do solo).

A comunidade vegetal do sapal de Pancas, estuário do Tejo, pobre em diversidade, apresenta uma baixa proporção de espécies vegetais que formam micorrizas (capítulo 2), como geralmente se verifica neste tipo de ecossistema (Tabela 1.1, Capítulo 1). É reconhecido que comunidades vegetais pertencentes a ecossistemas muito salinos ou húmidos contêm na sua maioria espécies que não formam associações do tipo micorriza (Allen, 1991; Peat & Fitter, 1993).

Nos sapais a baixa diversidade vegetal e a distribuição da vegetação são determinadas pelo alagamento e salinidade elevada dos solos em consequência da acção das marés (Long & Mason, 1983). Os resultados desta tese evidenciam que a presença, extensão da colonização e distribuição das AM num sapal parece ser determinada mais pela identidade das espécies vegetais, e consequentemente pela distribuição das plantas hospedeiras, do que por factores abióticos. Vários resultados apoiam esta afirmação: (i) semelhança no grau de colonização em plantas da mesma espécie, *Aster tripolium*, entre as zonas menos e mais alagadas, possuindo ambas as zonas idêntica composição na comunidade de fungos AM (Capítulo 2); (ii) presença de potencial de inóculo em ambas as zonas (Capítulos 2); (iii) relação entre a distribuição de propágulos de fungos AM e a distribuição das plantas hospedeiras (Capítulo 3); (iv) capacidade dos esporos de fungos AM germinarem perante níveis elevados de salinidade e água no solo (Capítulo 4); e (v) capacidade dos propágulos iniciarem a colonização de plantas sob condições de salinidade e alagamento (Capítulo 5). Estes resultados obtidos contrariam a sugestão feita por alguns autores de que a presença de AM nos sapais seria principalmente dependente das condições abióticas, sendo fortemente limitada nas zonas mais alagadas e salinas (van Duin *et al*., 1989; Cooke & Lefor, 1990).

Estudos efectuados noutros sapais do estuário do Tejo (Corroios e Rosário) que possuem salinidade mais elevada do que o sapal de Pancas, revelaram ausência de colonização AM nas plantas (*Spartima maritima*, *Halimione portulacoides*, *Arthrocnemum fruticosum* e *A. perenne*) e de esporos de fungos AM nos sedimentos (resultados não apresentados). A inexistência de espécies vegetais micotróficas nestes sapais não permite concluir se a ausência de AM foi consequência da incapacidade dos fungos AM de sobreviverem nessas condições abióticas extremas, ou unicamente da falta de espécies hospedeiras. Contudo, estudos realizados em dois sapais no estuário de Westerschelde, Holanda, com localizações e níveis de salinidade diferentes (Ritthem, sapal mais próximo do mar e com salinidade mais elevada, e Waarde, sapal mais distante do mar e com menor salinidade) revelaram que a colonização por fungos AM em *A. tripolium* não apresentava diferenças significativas entre os dois sapais, nem entre as zonas baixa e alta dentro de cada sapal (resultados não apresentados). Por outro lado, Brown & Bledsoe (1996) observaram níveis de colonização em zonas de sapal alagadas e com salinidade muito elevada (semelhante à da água do mar) semelhantes aos encontrados em zonas menos salinas e alagadas. Estas observações reforçam a importância da dominância de plantas não micotróficas ou de plantas formando níveis extremamente baixos de colonização, como factor determinante da quase ausência de AM em determinados sapais, ou zonas do sapal, sujeitos a maior frequência de alagamento salino.

Não é possível, no entanto, excluir completamente qualquer influência directa da salinidade na ocorrência de AM em sapais. Os resultados do Capítulo 5 mostram que o crescimento, actividade e extensão da colonização dos fungos AM de sapal são consideravelmente afectados por níveis elevados de salinidade, ao contrário do que se verifica com elevada intensidade de alagamento. Neste trabalho não foi possível concluir se a menor infectividade dos fungos AM sob condições salinas resultou de efeitos directos da salinidade no fungo ou do menor crescimento e tolerância das plantas de *A. tripolium* à salinidade. Estudos com outras espécies vegetais de sapal mais tolerantes à salinidade poderão elucidar se o desenvolvimento da associação micorrízica é mais limitado por efeitos directos da salinidade nos fungos AM do que por influência do hospedeiro, como verificado por Johnson-Green *et al*. (2001) noutro tipo de sistema salino.

Os sedimentos do sapal contêm potencial de inóculo capaz de iniciar a colonização (Capítulos 2 e 4), o que é condição fundamental na persistência de AM num determinado ecossistema (Brundrett, 1991). O menor número de propágulos presente na zona baixa do sapal comparativamente à zona alta (Capítulo 2), poderá resultar de menor esporulação (Aziz *et al*., 1995) e extensão do micélio extraradicular (Capítulo 5) sob condições de alagamento.

Todavia, o inóculo presente na zona de baixo deve ser suficiente para manter os níveis usuais de colonização nas plantas, uma vez que não foram detectadas diferenças significativas na colonização de *A. tripolium* entre as duas zonas (Capítulo 2). A distribuição do potencial de inóculo nos sapais é determinada principalmente pela distribuição das plantas hospedeiras (Capítulo 3), originando uma zonação no potencial de infectividade, que pode, provavelmente, dificultar a colonização de plantas fora dessas zonas, a não ser que ocorra dispersão de esporos. A presença de plantas de simbiose obrigatória em zonas de plantas não micotróficas poderá ser fortemente limitada pela ausência de propágulos de fungos AM, e assim, as AM poderão influenciar a distribuição da vegetação nos sapais.

A identificação das espécies de fungos AM através da análise morfológica de esporos revelou que os factores abióticos presentes no sapal terão influenciado a existência de um reduzido número de espécies (Capítulo 2 e 3), mas não seleccionaram a comunidade de fungos AM ao longo de um gradiente de alagamento salino (Capítulo 2). Este resultado difere do encontrado noutro tipo de sistemas muito alagados, em que a composição da comunidade fúngica variava com o gradiente hídrico (Miller & Bever, 1999). A identificação morfológica (Capítulos 2 e 3) juntamente com a identificação por métodos moleculares (Hildebrandt *et al*., 2001; Landwehr *et al*., 2002) indicam que algumas espécies de fungos AM, nomeadamente *Glomus geosporum*, a espécie mais abundante em vários sapais da Europa, são simbiontes com sucesso neste tipo de habitat.

O presente trabalho mostrou que a adaptação ecológica à salinidade e ao alagamento é um mecanismo explicativo da sobrevivência dos fungos AM nos sapais. O grau de tolerância e a capacidade de germinação e infecção dos fungos nativos do sapal a diferentes níveis de salinidade e de alagamento (Capítulos 4 e 5) explicam a sua ocorrência em diferentes zonas do sapal com frequências de alagamento salino distintas. Estas observações, juntamente com a taxa óptima de germinação a concentrações próximas das existentes no sapal estudado (Capítulo 4), sugerem a existência de ecótipos de fungos AM em sapais. Apesar de os fungos AM serem generalistas, existem evidências da existência de ecótipos em determinados habitats e da ocorrência de variação genética e fisiológica em populações de fungos AM da mesma espécie presentes em ambientes diferentes (ver Brundrett, 1991), o que poderá estar relacionado com a coexistência de genomas múltiplos nos fungos AM (Kuhn *et al*., 2001). Os resultados dos Capítulos 5 e 6 também permitiram mostrar que o alagamento tem pouco ou nenhum impacto na infectividade e actividade, respectivamente, dos fungos AM, salientando a provável adaptação *in situ* dos fungos nativos à inundação salina regular das marés.

A adaptação e a capacidade de infecção dos fungos AM nativos verificadas neste trabalho, em condições próprias do habitat, são requisitos importantes e mesmo essenciais para evidenciar o papel potencial que as micorrizas desempenham neste tipo de ecossistema.

Atendendo ao ambiente adverso em que vivem, as plantas de sapal necessitam de desenvolver adaptações para sobreviverem sob condições de alagamento periódico com água do mar. Os resultados do capítulo 6 mostram que os fungos AM podem aumentar a tolerância de *A. tripolium* do sapal de Pancas ao alagamento salino por acção da maré, promovendo o crescimento dos hospedeiros relativamente às plantas não micorrizadas. O facto de os fungos AM do sapal tolerarem o alagamento (Capítulos 4 e 5) e de *A. tripolium* estar sistematicamente colonizado, independentemente da época do ano, na zona baixa do sapal (Capítulo 2) e obter benefícios evidentes no seu crescimento sob condições de alagamento (Capítulo 6), indica que as AM desempenham papel importante na presença de *A. tripolium* na zona do sapal de Pancas mais frequentemente alagada.

Os efeitos benéficos das AM para as plantas de sapal, como noutros sistemas alagados, têm sido difíceis de demonstrar. O melhoramento da nutrição fosfatada, apontado como o principal benefício das AM para as plantas (Smith & Read, 1997), não se verificou em condições de alagamento nas plantas colonizadas de ambas as populações de *A. tripolium* estudadas (Capítulo 6). Tal facto não é surpreendente, uma vez que a importância das AM na tomada de P de espécies vegetais adaptadas a habitats ricos em fósforo, como acontece na maioria dos sapais, incluindo o de Pancas (Capítulo 2), é diminuta ou nula (Koide, 1991). Trabalhos anteriores mostraram a presença de AM em solos com altas concentrações de fósforo disponível e de fungos AM tolerantes ao fósforo (Sylvia & Schenck, 1983; Douds & Schenck, 1990) e a existência de benefícios das AM para as plantas em experiências com solos alagados com altos níveis de fósforo (Solaiman & Hirata, 1996, 1997).

Os resultados do Capítulo 6 mostraram que a indução pelas AM de alterações morfológicas e fisiológicas, ao nível do balanço funcional de carbono e da nutrição azotada, aumentam a tolerância da população de *A. tripolium* do estuário do Tejo ao alagamento salino. É também possível que as AM desempenhem papel na melhoria das relações hídricas das plantas (Augé, 2001), uma vez que nos sapais há reduzida de quantidade de água disponível para as plantas (Rozema *et al*., 1986).

A ausência de evidências de benefícios no crescimento das plantas de *A. tripolium* da população do estuário do Tejo em condições não alagadas (próximas das existentes na zona alta do sapal) e das plantas da população de Westerschelde (Capítulo 6), levanta a hipótese de se poder estar, nestes casos, perante uma relação de parasitismo ou comensalismo, em que os

custos para a planta em hidratos de carbono superam ou igualam, respectivamente, os benefícios de ter os fungos simbiontes. No entanto, o facto de as plantas de ambas as populações se encontrarem sempre micorrizadas na natureza, sugere que, pelo menos em determinadas fases do seu ciclo de vida, podem beneficiar da associação. Johnson *et al*. (1997) sugerem que em determinadas plantas ou sob determinadas circunstâncias fenológicas ou ambientais, pode haver um contínuo de parasitismo ou comensalismo a mutualismo nas associações micorrízicas. A ausência de benefícios no crescimento numa fase do ciclo de vida da planta não significa que não ocorram benefícios noutras etapas do ciclo, como sejam durante o período anterior à fase reprodutora ou na fase de maior produção de sementes (Koide *et al*., 1988; Lewis & Koide, 1990; Koide & Lu, 1991). A existência de maior grau de colonização em *A. tripolium* na zona alta do sapal de Pancas imediatamente antes e durante a floração e frutificação (Capítulo 2) parece estar de acordo com a sugestão anterior.

A ausência de benefícios verificada nas plantas da população de Westerschelde que tolerou o alagamento imposto, assim como a ocorrência de benefícios na população do Tejo apenas sob condições de alagamento (Capítulo 6), evidencia que os benefícios das AM no crescimento, pelo menos naquela fase do ciclo de vida de *A. tripolium*, são mediados por factores abióticos.

Uma maior tomada de azoto pelas plantas micorrizadas, independentemente da tomada de fósforo, do regime de alagamento e da população de *A. tripolium*, sugere que as AM podem desempenhar funções ao nível da nutrição azotada nos sapais. Sendo a disponibilidade de azoto, geralmente, um factor limitante para as plantas nos sapais, esta acção das AM, a confirmar-se, será relevante para a funcionalidade das plantas de sapal.

As AM podem proporcionar outros efeitos, não relacionados directamente com a tomada de nutrientes pelas plantas. Estudos recentes sugerem que os fungos AM influenciam as populações de bactérias do solo associado a *Spartina patens* com prováveis impactos no desempenho das plantas (Burke *et al*., 2002a,b, 2003). Por outro lado, interacções dos fungos AM com patogénicos assintomáticos da raiz podem fornecer protecção às plantas contra estes organismos, aumentando a sua fecundidade (Newsham *et al*., 1994, 1995).

As micorrizas são simbioses presentes na maioria dos habitats, mas a sua importância e função podem variar consoante os ecossistemas, dependendo das suas características abióticas e bióticas (Brundrett, 1991; Smith & Read, 1997). O trabalho desenvolvido nesta tese mostra que a presença de fungos AM, particularmente adaptados à salinidade e ao alagamento, constitui um factor importante na tolerância de algumas populações vegetais ao alagamento salino, tendo, muito provavelmente, o potencial de influenciar desse modo a distribuição das

espécies, e consequentemente, a estrutura das comunidades vegetais dos sapais, constituídas por uma mistura de plantas micotróficas e não micotróficas. O desenvolvimento de estudos sobre a influência das AM nas interacções competitivas entre espécies vegetais, particularmente entre espécies micotróficas e não micotróficas, relacionadas com a exploração dos recursos do solo, a nutrição mineral e a tolerância ao alagamento e à salinidade, será de extrema utilidade para aumentar a compreensão do significado ecológico das AM em sapais.

O potencial das AM para conferirem maior tolerância das plantas micotróficas ao alagamento salino, como se verifica com *A. tripolium* neste trabalho, pode ser um importante factor a ter em conta nos efeitos do aumento do nível de água do mar nos sapais que já acontece actualmente e se prevê para as próximas décadas. A rede de micélio extraradicular (Capítulos 4 e 5) ligando plantas vizinhas pode ter também importante acção na retenção de partículas, contribuindo para uma maior taxa de sedimentação que é fundamental para contrabalançar o aumento do nível da água do mar.

O facto do número de plantas micotróficas presente no sapal ser geralmente reduzido, não significa, por isso, que as associações micorrízicas não sejam relevantes na estrutura e funcionalidade das comunidades vegetais, à semelhança do que se verifica noutro tipo de ecossistemas (van der Heijden *et al*., 1998; O'Connor *et al*., 2002).

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